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(54) Title: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

(57) Abstract

Methods for preparing cell lines that contain artificial chromosomes, methods for preparation of artificial chromosomes, methods for purification of artificial chromosomes, methods for targeted insertion of heterologous DNA into artificial chromosomes, and methods for delivery of the chromosomes to selected cells and tissues are provided. Also provided are cell lines for use in the methods, and cell lines and chromosomes produced by the methods. In particular, satellite artificial chromosomes that, except for inserted heterologous DNA, are substantially composed of heterochromatin, are provided. Methods for use of the artificial chromosomes, including for gene therapy, production of gene products and production of transgenic plants and animals are also provided.

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ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

RELATED APPLICATIONS

For U.S. national stage purposes, this application is a continuation-

- 5 in-part of copending U.S. application Serial No. 08/695,191, filed August 7, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*. This application is also continuation-in-part of copending U.S. application Serial No. 08/682,080,
- 10 filed July 15, 1996 by GYULA HADLACZKY and ALADAR SZALAY, entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*, and is also a continuation-in-part of copending U.S. application Serial No. 08/629,822, filed April 10, 1996 by GYULA HADLACZKY and ALADAR SZALAY,
- 15 entitled *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*.

For international purposes, the benefit of priority to each of these application is claimed and the subject matter of that application is incorporated herein in its entirety.

- 20 U.S. application Serial No. 08/695,191 is a continuation-in-part of U.S. application Serial No. 08/682,080 and also is a continuation-in-part of U.S. application Serial No. 08/629,822. U.S. application Serial No. 08/682,080 is a continuation-in-part of U.S. application Serial No. 08/629,822.
- 25 This application is related to U.S. application Serial No. 07/759,558, now U.S. Patent No. 5,288,625, is related to U.S. application Serial No. 08/734,344, filed October 21, 1996, and is related to allowed U.S. application Serial No. 08/375,271, filed 1/19/95, which is a continuation of U.S. application Serial No. 08/080,097, filed 6/23/93

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which is a continuation of U.S. application Serial No. 07/892,487, filed 6/3/92, which is a continuation of U.S. application Serial No. 07/521,073, filed 5/9/90.

To the extent permitted, the subject matter of each of U.S.

- 5 application Serial Nos. 08/734,344, 08/695,191, 08/682,080,
08/629,822, 08/375,271, 08/080,097, 07/892,487, and 07/521,073,
and U.S. Patent No. 5,288,625 is incorporated in its entirety by
reference thereto.

FIELD OF THE INVENTION

- 10 The present invention relates to methods for preparing cell lines that contain artificial chromosomes, methods for isolation of the artificial chromosomes, targeted insertion of heterologous DNA into the chromosomes, delivery of the chromosomes to selected cells and tissues and methods for isolation and large-scale production of the
- 15 chromosomes. Also provided are cell lines for use in the methods, and cell lines and chromosomes produced by the methods. Further provided are cell-based methods for production of heterologous proteins, gene therapy methods and methods of generating transgenic animals, particularly non-human transgenic animals, that use artificial
- 20 chromosomes.

BACKGROUND OF THE INVENTION

Several viral vectors, non-viral, and physical delivery systems for gene therapy and recombinant expression of heterologous nucleic acids have been developed [see, e.g., Mitani *et al.* (1993) Trends Biotech.

- 25 11:162-166]. The presently available systems, however, have numerous limitations, particularly where persistent, stable, or controlled gene expression is required. These limitations include: (1) size limitations because there is a limit, generally on order of about ten kilobases [kB], at most, to the size of the DNA insert [gene] that can be accepted by viral

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vectors, whereas a number of mammalian genes of possible therapeutic importance are well above this limit, especially if all control elements are included; (2) the inability to specifically target integration so that random integration occurs which carries a risk of disrupting vital genes or cancer suppressor genes; (3) the expression of randomly integrated therapeutic genes may be affected by the functional compartmentalization in the nucleus and are affected by chromatin-based position effects; (4) the copy number and consequently the expression of a given gene to be integrated into the genome cannot be controlled. Thus, improvements in gene delivery and stable expression systems are needed [see, e.g., Mulligan (1993) Science **260**:926-932].

In addition, safe and effective vectors and gene therapy methods should have numerous features that are not assured by the presently available systems. For example, a safe vector should not contain DNA elements that can promote unwanted changes by recombination or mutation in the host genetic material, should not have the potential to initiate deleterious effects in cells, tissues, or organisms carrying the vector, and should not interfere with genomic functions. In addition, it would be advantageous for the vector to be non-integrative, or designed for site-specific integration. Also, the copy number of therapeutic gene(s) carried by the vector should be controlled and stable, the vector should secure the independent and controlled function of the introduced gene(s); and the vector should accept large (up to Mb size) inserts and ensure the functional stability of the insert.

The limitations of existing gene delivery technologies, however, argue for the development of alternative vector systems suitable for transferring large [up to Mb size or larger] genes and gene complexes together with regulatory elements that will provide a safe, controlled, and persistent expression of the therapeutic genetic material.

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At the present time, none of the available vectors fulfill all these requirements. Most of these characteristics, however, are possessed by chromosomes. Thus, an artificial chromosome would be an ideal vector for gene therapy, as well as for stable, high-level, controlled production

5 of gene products that require coordination of expression of numerous genes or that are encoded by large genes, and other uses. Artificial chromosomes for expression of heterologous genes in yeast are available, but construction of defined mammalian artificial chromosomes has not been achieved. Such construction has been hindered by the lack

10 of an isolated, functional, mammalian centromere and uncertainty regarding the requisites for its production and stable replication. Unlike in yeast, there are no selectable genes in close proximity to a mammalian centromere, and the presence of long runs of highly repetitive pericentric heterochromatic DNA makes the isolation of a mammalian centromere

15 using presently available methods, such as chromosome walking, virtually impossible. Other strategies are required for production of mammalian artificial chromosomes, and some have been developed. For example, U.S. Patent No. 5,288,625 provides a cell line that contains an artificial chromosome, a minichromosome, that is about 20 to 30

20 megabases. Methods provided for isolation of these chromosomes, however, provide preparations of only about 10-20% purity. Thus, development of alternative artificial chromosomes and perfection of isolation and purification methods as well as development of more versatile chromosomes and further characterization of the

25 minichromosomes is required to realize the potential of this technology.

Therefore, it is an object herein to provide mammalian artificial chromosomes and methods for introduction of foreign DNA into such chromosomes. It is also an object herein to provide methods of isolation and purification of the chromosomes. It is also an object herein to

- provide methods for introduction of the mammalian artificial chromosome into selected cells, and to provide the resulting cells, as well as transgenic non-human animals, birds, fish and plants that contain the artificial chromosomes. It is also an object herein to provide methods for
- 5 gene therapy and expression of gene products using artificial chromosomes. It is a further object herein to provide methods for constructing species-specific artificial chromosomes de novo. Another object herein is to provide methods to generate de novo mammalian artificial chromosomes.
- 10 **SUMMARY OF THE INVENTION**
- Mammalian artificial chromosomes [MACs] are provided. Also provided are artificial chromosomes for other higher eukaryotic species, such as insects, birds, fowl and fish, produced using the MACS and methods provided herein. Methods for generating and isolating such
- 15 chromosomes are provided. Methods using the MACs to construct artificial chromosomes from other species, such as insect, bird, fowl and fish species are also provided. The artificial chromosomes are fully functional stable chromosomes. Two types of artificial chromosomes are provided. One type, herein referred to as SATACs [satellite artificial
- 20 chromosomes or satellite DNA based artificial chromosomes (the terms are used interchangeably herein)] are stable heterochromatic chromosomes, and the other type are minichromosomes based on amplification of euchromatin.

Artificial chromosomes provide an extra-genomic locus for targeted

25 integration of megabase [Mb] pair size DNA fragments that contain single or multiple genes, including multiple copies of a single gene operatively linked to one promoter or each copy or several copies linked to separate promoters. Thus, methods using the MACs to introduce the genes into cells, tissues, and animals, as well as species such as birds, fowl, fish

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and plants, are also provided. The artificial chromosomes with integrated heterologous DNA may be used in methods of gene therapy, in methods of production of gene products, particularly products that require expression of multigenic biosynthetic pathways, and also are intended for
5 delivery into the nuclei of germline cells, such as embryo-derived stem cells [ES cells], for production of transgenic (non-human) animals, birds, fowl and fish. Transgenic plants, including monocots and dicots, are also contemplated herein.

Mammalian artificial chromosomes provide extra-genomic specific
10 integration sites for introduction of genes encoding proteins of interest and permit megabase size DNA integration so that, for example, genes encoding an entire metabolic pathway or a very large gene, such as the cystic fibrosis [CF; ~250 kb] genomic DNA gene, several genes, such as multiple genes encoding a series of antigens for preparation of a
15 multivalent vaccine, can be stably introduced into a cell. Vectors for targeted introduction of such genes, including the tumor suppressor genes, such as p53, the cystic fibrosis transmembrane regulator cDNA [CFTR], and the genes for anti-HIV ribozymes, such as an anti-HIV gag ribozyme gene, into the artificial chromosomes are also provided.
20 The chromosomes provided herein are generated by introducing heterologous DNA that includes DNA encoding one or multiple selectable marker(s) into cells, preferably a stable cell line, growing the cells under selective conditions, and identifying from among the resulting clones those that include chromosomes with more than one centromere and/or
25 fragments thereof. The amplification that produces the additional centromere or centromeres occurs in cells that contain chromosomes in which the heterologous DNA has integrated near the centromere in the pericentric region of the chromosome. The selected clonal cells are then used to generate artificial chromosomes.

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Although non-targeted introduction of DNA, which results in some frequency of integration into appropriate loci, targeted introduction is preferred. Hence, in preferred embodiments, the DNA with the selectable marker that is introduced into cells to initiate generation of

- 5 artificial chromosomes includes sequences that target it to the an amplifiable region, such as the pericentric region, heterochromatin, and particularly rDNA of the chromosome. For example, vectors, such as pTEMPUD and pHASPUD [provided herein], which include such DNA specific for mouse satellite DNA and human satellite DNA, respectively,
- 10 are provided. The plasmid pHASPUD is a derivative of pTEMPUD that contains human satellite DNA sequences that specifically target human chromosomes. Preferred targeting sequences include mammalian ribosomal RNA (rRNA) gene sequences (referred to herein as rDNA) which target the heterologous DNA to integrate into the rDNA region of
- 15 those chromosomes that contain rDNA. For example, vectors, such as pTERPUD, which include mouse rDNA, are provided. Upon integration into existing chromosomes in the cells, these vectors can induce the amplification that results in generation of additional centromeres.

- Artificial chromosomes are generated by culturing the cells with
- 20 the multicentric, typically dicentric, chromosomes under conditions whereby the chromosome breaks to form a minichromosome and formerly dicentric chromosome. Among the MACs provided herein are the SATACs, which are primarily made up of repeating units of short satellite DNA and are nearly fully heterochromatic, so that without
 - 25 insertion of heterologous or foreign DNA, the chromosomes preferably contain no genetic information or contain only non-protein-encoding gene sequences such as rDNA sequences. They can thus be used as "safe" vectors for delivery of DNA to mammalian hosts because they do not contain any potentially harmful genes. The SATACs are generated, not

from the minichromosome fragment as, for example, in U.S. Patent No. 5,288,625, but from the fragment of the formerly dicentric chromosome.

- In addition, methods for generating euchromatic minichromosomes and the use thereof are also provided herein. Methods for generating 5 one type of MAC, the minichromosome, previously described in U.S. Patent No. 5,288,625, and the use thereof for expression of heterologous DNA are provided. In a particular method provided herein for generating a MAC, such as a minichromosome, heterologous DNA that includes mammalian rDNA and one or more selectable marker genes 10 is introduced into cells which are then grown under selective conditions. Resulting cells that contain chromosomes with more than one centromere are selected and cultured under conditions whereby the chromosome breaks to form a minichromosome and a formerly multicentric (typically dicentric) chromosome from which the minichromosome was released.
- 15 Cell lines containing the minichromosome and the use thereof for cell fusion are also provided. In one embodiment, a cell line containing the mammalian minichromosome is used as recipient cells for donor DNA encoding a selected gene or multiple genes. To facilitate integration of the donor DNA into the minichromosome, the recipient cell line preferably 20 contains the minichromosome but does not also contain the formerly dicentric chromosome. This may be accomplished by methods disclosed herein such as cell fusion and selection of cells that contain a minichromosome and no formerly dicentric chromosome. The donor DNA is linked to a second selectable marker and is targeted to and integrated 25 into the minichromosome. The resulting chromosome is transferred by cell fusion into an appropriate recipient cell line, such as a Chinese hamster cell line [CHO]. After large-scale production of the cells carrying the engineered chromosome, the chromosome is isolated. In particular, metaphase chromosomes are obtained, such as by addition of colchicine,

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and they are purified from the cell lysate. These chromosomes are used for cloning, sequencing and for delivery of heterologous DNA into cells.

Also provided are SATACs of various sizes that are formed by repeated culturing under selective conditions and subcloning of cells that

- 5 contain chromosomes produced from the formerly dicentric chromosomes. The exemplified SATACs are based on repeating DNA units that are about 15 Mb [two ~ 7.5 Mb blocks]. The repeating DNA unit of SATACs formed from other species and other chromosomes may vary, but typically would be on the order of about 7 to about 20 Mb.
- 10 The repeating DNA units are referred to herein as megareplicons, which in the exemplified SATACs contain tandem blocks of satellite DNA flanked by non-satellite DNA, including heterologous DNA and non-satellite DNA. Amplification produces an array of chromosome segments [each called an amplicon] that contain two inverted megareplicons
- 15 bordered by heterologous ["foreign"] DNA. Repeated cell fusion, growth on selective medium and/or BrdU [5-bromodeoxyuridine] treatment or other treatment with other genome destabilizing reagent or agent, such as ionizing radiation, including X-rays, and subcloning results in cell lines that carry stable heterochromatic or partially heterochromatic
- 20 chromosomes, including a 150-200 Mb "sausage" chromosome, a 500-1000 Mb gigachromosome, a stable 250-400 Mb megachromosome and various smaller stable chromosomes derived therefrom. These chromosomes are based on these repeating units and can include heterologous DNA that is expressed.
- 25 Thus, methods for producing MACs of both types (i.e., SATACs and minichromosomes) are provided. These methods are applicable to the production of artificial chromosomes containing centromeres derived from any higher eukaryotic cell, including mammals, birds, fowl, fish, insects and plants.

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The resulting chromosomes can be purified by methods provided herein to provide vectors for introduction of heterologous DNA into selected cells for production of the gene product(s) encoded by the heterologous DNA, for production of transgenic (non-human) animals,

- 5 birds, fowl, fish and plants or for gene therapy.

In addition, methods and vectors for fragmenting the minichromosomes and SATACs are provided. Such methods and vectors can be used for in vivo generation of smaller stable artificial chromosomes. Vectors for chromosome fragmentation are used to

- 10 produce an artificial chromosome that contains a megareplicon, a centromere and two telomeres and will be between about 7.5 Mb and about 60 Mb, preferably between about 10 Mb-15 Mb and 30-50 Mb. As exemplified herein, the preferred range is between about 7.5 Mb and 50 Mb. Such artificial chromosomes may also be produced by other
15 methods.

Isolation of the 15 Mb [or 30 Mb amplicon containing two 15 Mb inverted repeats] or a 30 Mb or higher multimer, such as 60 Mb, thereof should provide a stable chromosomal vector that can be manipulated in vitro. Methods for reducing the size of the MACs to generate smaller
20 stable self-replicating artificial chromosomes are also provided.

Also provided herein, are methods for producing mammalian artificial chromosomes, including those provided herein, in vitro, and the resulting chromosomes. The methods involve *in vitro* assembly of the structural and functional elements to provide a stable artificial
25 chromosome. Such elements include a centromere, two telomeres, at least one origin of replication and filler heterochromatin, e.g., satellite DNA. A selectable marker for subsequent selection is also generally included. These specific DNA elements may be obtained from the artificial chromosomes provided herein such as those that have been

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generated by the introduction of heterologous DNA into cells and the subsequent amplification that leads to the artificial chromosome, particularly the SATACs. Centromere sequences for use in the *in vitro* construction of artificial chromosomes may also be obtained by

- 5 employing the centromere cloning methods provided herein. In preferred embodiments, the sequences providing the origin of replication, in particular, the megareplicator, are derived from rDNA. These sequences preferably include the rDNA origin of replication and amplification promoting sequences.

10 Methods and vectors for targeting heterologous DNA into the artificial chromosomes are also provided as are methods and vectors for fragmenting the chromosomes to produce smaller but stable and self-replicating artificial chromosomes.

- The chromosomes are introduced into cells to produce stable
15 transformed cell lines or cells, depending upon the source of the cells. Introduction is effected by any suitable method including, but not limited to electroporation, direct uptake, such as by calcium phosphate precipitation, uptake of isolated chromosomes by lipofection, by microc II fusion, by lipid-mediated carrier systems or other suitable method. The
20 resulting cells can be used for production of proteins in the cells. The chromosomes can be isolated and used for gene delivery. Methods for isolation of the chromosomes based on the DNA content of the chromosomes, which differs in MACs versus the authentic chromosomes, are provided. Also provided are methods that rely on
25 content, particularly density, and size of the MACs.

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These artificial chromosomes can be used in gene therapy, gene product production systems, production of humanized genetically transformed animal organs, production of transgenic plants and animals (non-human), including mammals, birds, fowl, fish, invertebrates,

5 vertebrates, reptiles and insects, any organism or device that would employ chromosomal elements as information storage vehicles, and also for analysis and study of centromere function, for the production of artificial chromosome vectors that can be constructed in vitro, and for the preparation of species-specific artificial chromosomes. The artificial

10 chromosomes can be introduced into cells using microinjection, cell fusion, microcell fusion, electroporation, nuclear transfer, electrofusion, projectile bombardment, nuclear transfer, calcium phosphate precipitation, lipid-mediated transfer systems and other such methods. Cells particularly suited for use with the artificial chromosomes include,

15 but are not limited to plant cells, particularly tomato, arabidopsis, and others, insect cells, including silk worm cells, insect larvae, fish, reptiles, amphibians, arachnids, mammalian cells, avian cells, embryonic stem cells, haematopoietic stem cells, embryos and cells for use in methods of genetic therapy, such as lymphocytes that are used in methods of adoptive immunotherapy and nerve or neural cells. Thus methods of producing gene products and transgenic (non-human) animals and plants are provided. Also provided are the resulting transgenic animals and plants.

20

Exemplary cell lines that contain these chromosomes are also provided.

25 Methods for preparing artificial chromosomes for particular species and for cloning centromeres are also provided. For example, two exemplary methods provided for generating artificial chromosomes for use in different species are as follows. First, the methods herein may be applied to different species. Second, means for generating species-

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- specific artificial chromosomes and for cloning centromeres are provided. In particular, a method for cloning a centromere from an animal or plant is provided by preparing a library of DNA fragments that contain the genome of the plant or animal and introducing each of the fragments
- 5 into a mammalian satellite artificial chromosome [SATAc] that contains a centromere from a species, generally a mammal, different from the selected plant or animal, generally a non-mammal, and a selectable marker. The selected plant or animal is one in which the mammalian species centromere does not function. Each of the SATAcs is
- 10 introduced into the cells, which are grown under selective conditions, and cells with SATACs are identified. Such SATACS should contain a centromere encoded by the DNA from the library or should contain th necessary elements for stable replication in the selected species.
- 15 Also provided are libraries in which the relatively large fragments of DNA are contained on artificial chromosomes.
- Transgenic (non-human) animals, invertebrates and vertebrates, plants and insects, fish, reptiles, amphibians, arachnids, birds, fowl, and mammals are also provided. Of particular interest are transgenic (non-human) animals and plants that express genes that confer resistance or
- 20 reduce susceptibility to disease. For example, the transgene may encode a protein that is toxic to a pathogen, such as a virus, bacterium or pest, but that is not toxic to the transgenic host. Furthermore, since multiple genes can be introduced on a MAC, a series of genes encoding an antigen can be introduced, which upon expression will serve to
- 25 immunize [in a manner similar to a multivalent vaccine] the host animal against the diseases for which exposure to the antigens provide immunity or some protection.

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Also of interest are transgenic (non-human) animals that serve as models of certain diseases and disorders for use in studying the disease and developing therapeutic treatments and cures thereof. Such animal models of disease express genes [typically carrying a disease-associated mutation], which are introduced into the animal on a MAC and which induce the disease or disorder in the animal. Similarly, MACs carrying genes encoding antisense RNA may be introduced into animal cells to generate conditional "knock-out" transgenic (non-human) animals. In such animals, expression of the antisense RNA results in decreased or complete elimination of the products of genes corresponding to the antisense RNA. Of further interest are transgenic mammals that harbor MAC-carried genes encoding therapeutic proteins that are expressed in the animal's milk. Transgenic (non-human) animals for use in xenotransplantation, which express MAC-carried genes that serve to humanize the animal's organs, are also of interest. Genes that might be used in humanizing animal organs include those encoding human surface antigens.

Methods for cloning centromeres, such as mammalian centromeres, are also provided. In particular, in one embodiment, a library composed of fragments of SATACs are cloned into YACs [yeast artificial chromosomes] that include a detectable marker, such as DNA encoding tyrosinase, and then introduced into mammalian cells, such as albino mouse embryos. Mice produced from embryos containing such YACs that include a centromere that functions in mammals will express the detectable marker. Thus, if mice are produced from albino mouse embryos into which a functional mammalian centromere was introduced, the mice will be pigmented or have regions of pigmentation.

A method for producing repeated tandem arrays of DNA is provided. This method, exemplified herein using telomeric DNA, is applicable to any repeat sequence, and in particular, low complexity repeats. The method provided herein for synthesis of arrays of tandem

5 DNA repeats are based in a series of extension steps in which successive doublings of a sequence of repeats results in an exponential expansion of the array of tandem repeats. An embodiment of the method of synthesizing DNA fragments containing tandem repeats may generally be described as follows. Two oligonucleotides are used as starting

10 materials. Oligonucleotide 1 is of length k of repeated sequence (the flanks of which are not relevant) and contains a relatively short stretch (60-90 nucleotides) of the repeated sequence, flanked with appropriately chosen restriction sites:-

15 where S1 is restriction site 1 cleaved by E1, S2 is a second restriction site cleaved by E2 > represents a simple repeat unit, and '—' denotes a short (8-10) nucleotide flanking sequence complementary to oligonucleotide 2:

3'- S3-5'

20 where S3 is a third restriction site for enzyme E3 and which is present in the vector to be used during the construction. The method involves the following steps: (1) oligonucleotides 1 and 2 are annealed; (2) the annealed oligonucleotides are filled-in to produce a double-stranded (ds) sequence; (3) the double-stranded DNA is cleaved with restriction enzymes E1 and E3 and subsequently ligated into a vector (e.g., pUC19 or a yeast vector) that has been cleaved with the same enzymes E1 and E3; (4) the insert is isolated from a first portion of the plasmid by digesting with restriction enzymes E1 and E3, and a second portion of the plasmid is cut with enzymes E2 (treated to remove the 3'-overhang)

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- and E3, and the large fragment (plasmid DNA plus the insert) is isolated; (5) the two DNA fragments (the S1-S3 insert fragment and the vector plus insert) are ligated; and (6) steps 4 and 5 are repeated as many times as needed to achieve the desired repeat sequence size. In each
- 5 extension cycle, the repeat sequence size doubles, i.e., if m is the number of extension cycles, the size of the repeat sequence will be k x 2^m nucleotides.

DESCRIPTION OF THE DRAWINGS

- Figure 1 is a schematic drawing depicting formation of the
- 10 MMCneo [the minichromosome] chromosome. A-G represents the successive events consistent with observed data that would lead to the formation and stabilization of the minichromosome.

- Figure 2 shows a schematic summary of the manner in which the observed new chromosomes would form, and the relationships among
- 15 the different *de novo* formed chromosomes. In particular, this figure shows a schematic drawing of the *de novo* chromosome formation initiated in the centromeric region of mouse chromosome 7. (A) A single E-type amplification in the centromeric region of chromosome 7 generates a neo-centromere linked to the integrated "foreign" DNA, and
- 20 forms a dicentric chromosome. Multiple E-type amplification forms the neo-chromosome, which separates from the remainder of mouse chromosome 7 through a specific breakage between the centromeres of the dicentric chromosome and which was stabilized in a mouse-hamster hybrid cell line; (B) Specific breakage between the centromeres of a
- 25 dicentric chromosome 7 generates a chromosome fragment with the neo-centromere, and a chromosome 7 with traces of heterologous DNA at the end; (C) Inverted duplication of the fragment bearing the neo-centromere results in the formation of a stable neo-minichromosome; (D) Integration of exogenous DNA into the heterologous DNA region of the form

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- dicentric chromosome 7 initiates H-type amplification, and the formation of a heterochromatic arm. By capturing a euchromatic terminal segment, this new chromosome arm is stabilized in the form of the "sausage" chromosome; (E) BrdU [5-bromodeoxyuridine] treatment and/or drug selection induce further H-type amplification, which results in the formation of an unstable gigachromosome; (F) Repeated BrdU treatments and/or drug selection induce further H-type amplification including a centromere duplication, which leads to the formation of another heterochromatic chromosome arm. It is split off from the chromosome 7 by chromosome breakage, and by acquiring a terminal segment, the stable megachromosome is formed.

Figure 3 is a schematic diagram of the replicon structure and a scheme by which a megachromosome could be produced.

- Figure 4 sets forth the relationships among some of the exemplary cell lines described herein.

Figure 5 is a diagram of the plasmid pTEMPUD.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

- Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference.

As used herein, a mammalian artificial chromosome [MAC] is a piece of DNA that can stably replicate and segregate alongside endogenous chromosomes. It has the capacity to accommodate and express heterologous genes inserted therein. It is referred to as a mammalian artificial chromosome because it includes an active mammalian centromere(s). Plant artificial chromosomes, insect artificial chromosomes and avian artificial chromosomes refer to chromosomes

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that include plant and insect centromeres, respectively. A human artificial chromosome [HAC] refers to chromosomes that include human centromeres, BUGACs refer to insect artificial chromosomes, and AVACs refer to avian artificial chromosomes. Among the MACs provided herein 5 are SATACs, minichromosomes, and in vitro synthesized artificial chromosomes. Methods for construction of each type are provided herein.

As used herein, in vitro synthesized artificial chromosomes are artificial chromosomes that is produced by joining the essential 10 components (at least the centromere, and origins of replication) in vitro.

As used herein, endogenous chromosomes refer to genomic chromosomes as found in the cell prior to generation or introduction of a MAC.

As used herein, stable maintenance of chromosomes occurs when 15 at least about 85%, preferably 90%, more preferably 95%, of the cells retain the chromosome. Stability is measured in the presence of a selective agent. Preferably these chromosomes are also maintained in the absence of a selective agent. Stable chromosomes also retain their structure during cell culturing, suffering neither intrachromosomal nor 20 interchromosomal rearrangements.

As used herein, growth under selective conditions means growth of a cell under conditions that require expression of a selectable marker for survival.

As used herein, an agent that destabilizes a chromosome is any 25 agent known by those of skill in the art to enhance amplification events, mutations. Such agents, which include BrdU, are well known to those of skill in the art.

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As used herein, *de novo* with reference to a centromere, refers to generation of an excess centromere as a result of incorporation of a heterologous DNA fragment using the methods herein.

As used herein, euchromatin and heterochromatin have their

- 5 recognized meanings, euchromatin refers to chromatin that stains diffusely and that typically contains genes, and heterochromatin refers to chromatin that remains unusually condensed and that has been thought to be transcriptionally inactive. Highly repetitive DNA sequences [satellite DNA], at least with respect to mammalian cells, are usually
- 10 located in regions of the heterochromatin surrounding the centromere [pericentric heterochromatin]. Constitutive heterochromatin refers to heterochromatin that contains the highly repetitive DNA which is constitutively condensed and genetically inactive.

As used herein, BrdU refers to 5-bromodeoxyuridine, which during replication is inserted in place of thymidine. BrdU is used as a mutagen; it also inhibits condensation of metaphase chromosomes during cell division.

As used herein, a dicentric chromosome is a chromosome that contains two centromeres. A multicentric chromosome contains more than two centromeres.

As used herein, a formerly dicentric chromosome is a chromosome that is produced when a dicentric chromosome fragments and acquires new telomeres so that two chromosomes, each having one of the centromeres, are produced. Each of the fragments are replicable

- 25 chromosomes. If one of the chromosomes undergoes amplification of euchromatic DNA to produce a fully functional chromosome that contains the newly introduced heterologous DNA and primarily [at least more than 50%] euchromatin, it is a minichromosome. The remaining chromosome is a formerly dicentric chromosome. If one of the chromosomes

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undergoes amplification, whereby heterochromatin [satellite DNA] is amplified and a euchromatic portion [or arm] remains, it is referred to as a sausage chromosome. A chromosome that is substantially all heterochromatin, except for portions of heterologous DNA, is called a

- 5 SATAc. Such chromosomes [SATAcs] can be produced from sausage chromosomes by culturing the cell containing the sausage chromosome under conditions, such as BrdU treatment and/or growth under selective conditions, that destabilize the chromosome so that a satellite artificial chromosomes [SATAc] is produced. For purposes herein, it is
- 10 understood that SATAcs may not necessarily be produced in multiple steps, but may appear after the initial introduction of the heterologous DNA and growth under selective conditions, or they may appear after several cycles of growth under selective conditions and BrdU treatment.

As used herein, a SATAc refers to a chromosome that is

- 15 substantially all heterochromatin, except for portions of heterologous DNA. Typically, SATAcs are satellite DNA based artificial chromosomes, but the term encompasses any chromosome made by the methods herein that contains more heterochromatin than euchromatin.

As used herein, amplifiable, when used in reference to a

- 20 chromosome, particularly the method of generating SATAcs provided herein, refers to a region of a chromosome that is prone to amplification. Amplification typically occurs during replication and other cellular events involving recombination. Such regions are typically regions of the chromosome that include tandem repeats, such as satellite DNA, rDNA
- 25 and other such sequences.

As used herein, amplification, with reference to DNA, is a process in which segments of DNA are duplicated to yield two or multiple copies of identical or nearly identical DNA segments that are typically joined as substantially tandem or successive repeats or inverted repeats.

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As used herein an amplicon is a repeated DNA amplification unit that contains a set of inverted repeats of the megareplicon. A megareplicon represents a higher order replication unit. For example, with reference to the SATACs, the megareplicon contains a set of 5 tandem DNA blocks each containing satellite DNA flanked by non-satellite DNA. Contained within the megareplicon is a primary replication site, referred to as the megareplicator, which may be involved in organizing and facilitating replication of the pericentric heterochromatin and possibly the centromeres. Within the megareplicon there may be 10 smaller [e.g., 50-300 kb in some mammalian cells] secondary replicons. In the exemplified SATACS, the megareplicon is defined by two tandem ~ 7.5 Mb DNA blocks [see, e.g., Fig. 3]. Within each artificial chromosome [AC] or among a population thereof, each amplicon has the same gross structure but may contain sequence variations. Such 15 variations will arise as a result of movement of mobile genetic elements, deletions or insertions or mutations that arise, particularly in culture. Such variation does not affect the use of the ACs or their overall structure as described herein.

As used herein, ribosomal RNA [rRNA] is the specialized RNA that 20 forms part of the structure of a ribosome and participates in the synthesis of proteins. Ribosomal RNA is produced by transcription of genes which, in eukaryotic cells, are present in multiple copies. In human cells, the approximately 250 copies of rRNA genes per haploid genome are spread out in clusters on at least five different chromosomes 25 (chromosomes 13, 14, 15, 21 and 22). In mouse cells, the presence of ribosomal DNA [rDNA] has been verified on at least 11 pairs out of 20 mouse chromosomes [chromosomes 5, 6, 9, 11, 12, 15, 16, 17, 18, 19 and X][see .g., Rowe et al. (1996) Mamm. Genome 7:886-889 and Johnson et al. (1993) Mamm. Genome 4:49-52]. In eukaryotic cells, the

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multiple copies of the highly conserved rRNA genes are located in a tandemly arranged series of rDNA units, which are generally about 40-45 kb in length and contain a transcribed region and a nontranscribed region known as spacer (*i.e.*, intergenic spacer) DNA which can vary in length
5 and sequence. In the human and mouse, these tandem arrays of rDNA units are located adjacent to the pericentric satellite DNA sequences (heterochromatin). The regions of these chromosomes in which the rDNA is located are referred to as nucleolar organizing regions (NOR) which loop into the nucleolus, the site of ribosome production within the
10 cell nucleus.

As used herein, the minichromosome refers to a chromosome derived from a multicentric, typically dicentric, chromosome [see, e.g., FIG. 1] that contains more euchromatic than heterochromatic DNA.

As used herein, a megachromosome refers to a chromosome that,
15 except for introduced heterologous DNA, is substantially composed of heterochromatin. Megachromosomes are made of an array of repeated amplicons that contain two inverted megareplicons bordered by introduced heterologous DNA [see, e.g., Figure 3 for a schematic drawing of a megachromosome]. For purposes herein, a
20 megachromosome is about 50 to 400 Mb, generally about 250-400 Mb. Shorter variants are also referred to as truncated megachromosomes [about 90 to 120 or 150 Mb], dwarf megachromosomes [\sim 150-200 Mb] and cell lines, and a micro-megachromosome [\sim 50-90 Mb, typically 50-60 Mb]. For purposes herein, the term megachromosome refers to the
25 overall repeated structure based on an array of repeated chromosomal segments [amplicons] that contain two inverted megareplicons bordered by any inserted heterologous DNA. The size will be specified.

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- As used herein, genetic therapy involves the transfer or insertion of heterologous DNA into certain cells, target cells, to produce specific gene products that are involved in correcting or modulating disease. The DNA is introduced into the selected target cells in a manner such that the
- 5 heterologous DNA is expressed and a product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product. It may encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product.
- 10 Genetic therapy may also be used to introduce therapeutic compounds, such as TNF, that are not normally produced in the host or that are not produced in therapeutically effective amounts or at a therapeutically useful time. Expression of the heterologous DNA by the target cells within an organism afflicted with the disease thereby enables modulation
- 15 of the disease. The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

- As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally
- 20 as part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. It is DNA or RNA that is not endogenous to the cell and has been exogenously introduced into the cell. Examples of heterologous DNA include, but are not limited to, DNA that encodes a
- 25 gene product or gene product(s) of interest, introduced for purposes of gene therapy or for production of an encoded protein. Other examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-

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cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

- 5 As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures said disease.
- 10 As used herein, transgenic plants refer to plants in which heterologous or foreign DNA is expressed or in which the expression of a gene naturally present in the plant has been altered.
- As used herein, operative linkage of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters,
- 15 enhancers, transcriptional and translational stop sites, and other signal sequences refers to the relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical relationship between the DNA and the promoter such that the transcription of such
- 20 DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame. Preferred promoters include tissue specific promoters, such as mammary gland specific promoters, viral promoters, such TK, CMV, adenovirus promoters, and other promoters known to those of skill in the
- 25 art.

As used herein, isolated, substantially pure DNA refers to DNA fragments purified according to standard techniques employed by those skilled in the art, such as that found in Maniatis *et al.* [(1982) Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY].

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into peptides, polypeptides, 5 or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression of 10 the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, transformation/transfection refers to the process by which DNA or RNA is introduced into cells. Transfection refers to 15 the taking up of exogenous nucleic acid, e.g., an expression vector, by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, by direct uptake using calcium phosphate [CaPO₄; see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:1373-20 1376], polyethylene glycol [PEG]-mediated DNA uptake, electroporation, lipofection [see, e.g., Strauss (1996) Meth. Mol. Biol. 54:307-327], microcell fusion [see, EXAMPLES, see, also Lambert (1991) Proc. Natl. Acad. Sci. U.S.A. 88:5907-5911; U.S. Patent No. 5,396,767, Sawford et al. (1987) Somatic Cell Mol. Genet. 13:279-284; Dhar et al. (1984) 25 Somatic Cell Mol. Genet. 10:547-559; and McNeill-Killary et al. (1995) Meth. Enzymol. 254:133-152], lipid-mediated carrier systems [see, e.g., Teifel et al. (1995) Biotechniques 19:79-80; Albrecht et al. (1996) Ann. Hematol. 72:73-79; Holmen et al. (1995) In Vitro Cell Dev. Biol. Anim. 31:347-351; REmy et al. (1994) Bioconjug. Chem. 5:647-654; Le Bolch

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et al. (1995) Tetrahedron Lett. 36:6681-6684; Loeffler et al. (1993) Meth. Enzymol. 217:599-618] or other suitable method. Successful transfection is generally recognized by detection of the presence of the heterologous nucleic acid within the transfected cell, such as any

5 indication of the operation of a vector within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration.

As used herein, injected refers to the microinjection [use of a small
10 syringe] of DNA into a cell.

As used herein, substantially homologous DNA refers to DNA that includes a sequence of nucleotides that is sufficiently similar to another such sequence to form stable hybrids under specified conditions.

It is well known to those of skill in this art that nucleic acid
15 fragments with different sequences may, under the same conditions, hybridize detectably to the same "target" nucleic acid. Two nucleic acid fragments hybridize detectably, under stringent conditions over a sufficiently long hybridization period, because one fragment contains a segment of at least about 14 nucleotides in a sequence which is
20 complementary [or nearly complementary] to the sequence of at least one segment in the other nucleic acid fragment. If the time during which hybridization is allowed to occur is held constant, at a value during which, under preselected stringency conditions, two nucleic acid fragments with exactly complementary base-pairing segments hybridize
25 detectably to each other, departures from exact complementarity can be introduced into the base-pairing segments, and base-pairing will nonetheless occur to an extent sufficient to make hybridization detectable. As the departure from complementarity between the base-pairing segments of two nucleic acids becomes larger, and as conditions

of the hybridization become more stringent, the probability decreases that the two segments will hybridize detectably to each other.

- Two single-stranded nucleic acid segments have "substantially the same sequence," within the meaning of the present specification, if
- 5 (a) both form a base-paired duplex with the same segment, and (b) the melting temperatures of said two duplexes in a solution of 0.5 X SSPE differ by less than 10°C. If the segments being compared have the same number of bases, then to have "substantially the same sequence", they will typically differ in their sequences at fewer than 1 base in 10.
- 10 Methods for determining melting temperatures of nucleic acid duplexes are well known [see, e.g., Meinkoth and Wahl (1984) Anal. Biochem. 138:267-284 and references cited therein].
- As used herein, a nucleic acid probe is a DNA or RNA fragment that includes a sufficient number of nucleotides to specifically hybridize
- 15 to DNA or RNA that includes identical or closely related sequences of nucleotides. A probe may contain any number of nucleotides, from as few as about 10 and as many as hundreds of thousands of nucleotides. The conditions and protocols for such hybridization reactions are well known to those of skill in the art as are the effects of probe size,
- 20 temperature, degree of mismatch, salt concentration and other parameters on the hybridization reaction. For example, the lower the temperature and higher the salt concentration at which the hybridization reaction is carried out, the greater the degree of mismatch that may be present in the hybrid molecules.
- 25 To be used as a hybridization probe, the nucleic acid is generally rendered detectable by labelling it with a detectable moiety or label, such as ³²P, ³H and ¹⁴C, or by other means, including chemical labelling, such as by nick-translation in the presence of deoxyuridylate biotinylated at the 5'-position of the uracil moiety. The resulting probe includes the

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- biotinylated uridylate in place of thymidylate residues and can be detected [via the biotin moieties] by any of a number of commercially available detection systems based on binding of streptavidin to the biotin. Such commercially available detection systems can be obtained, for
- 5 example, from Enzo Biochemicals, Inc. [New York, NY]. Any other label known to those of skill in the art, including non-radioactive labels, may be used as long as it renders the probes sufficiently detectable, which is a function of the sensitivity of the assay, the time available [for culturing cells, extracting DNA, and hybridization assays], the quantity of DNA or
- 10 RNA available as a source of the probe, the particular label and the means used to detect the label.

- Once sequences with a sufficiently high degree of homology to the probe are identified, they can readily be isolated by standard techniques, which are described, for example, by Maniatis *et al.* ((1982) Molecular
- 15 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

- As used herein, conditions under which DNA molecules form stable hybrids and are considered substantially homologous are such that DNA molecules with at least about 60% complementarity form stable
- 20 hybrids. Such DNA fragments are herein considered to be "substantially homologous". For example, DNA that encodes a particular protein is substantially homologous to another DNA fragment if the DNA forms stable hybrids such that the sequences of the fragments are at least about 60% complementary and if a protein encoded by the DNA retains
- 25 its activity.

For purposes herein, the following stringency conditions are defined:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C
- 2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C

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3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C
or any combination of salt and temperature and other reagents that result
in selection of the same degree of mismatch or matching.

As used herein, immunoprotective refers to the ability of a vaccine
5 or exposure to an antigen or immunity-inducing agent, to confer upon a
host to whom the vaccine or antigen is administered or introduced, the
ability to resist infection by a disease-causing pathogen or to have
reduced symptoms. The selected antigen is typically an antigen that is
presented by the pathogen.

10 As used herein, all assays and procedures, such as hybridization
reactions and antibody-antigen reactions, unless otherwise specified, are
conducted under conditions recognized by those of skill in the art as
standard conditions.

A. Preparation of cell lines containing MACs

15 1. The megareplicon

The methods, cells and MACs provided herein are produced by
virtue of the discovery of the existence of a higher-order replication unit
[megareplicon] of the centromeric region. This megareplicon is delimited
by a primary replication initiation site [megareplicator], and appears to
20 facilitate replication of the centromeric heterochromatin, and most likely,
centromeres. Integration of heterologous DNA into the megareplicator
region or in close proximity thereto, initiates a large-scale amplification of
megabase-size chromosomal segments, which leads to *de novo*
chromosome formation in living cells.

25 DNA sequences that provide a preferred megareplicator are the
rDNA units that give rise to ribosomal RNA (rRNA). In mammals,
particularly mice and humans, these rDNA units contain specialized
elements, such as the origin of replication (or origin of bidirectional
replication, i.e., OBR, in mouse) and amplification promoting sequences

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- (APS) and amplification control elements (ACE) (see, e.g., Gogel *et al.* (1996) Chromosoma 104:511-518; Coffman *et al.* (1993) Exp. Cell. Res. 209:123-132; Little *et al.* (1993) Mol. Cell. Biol. 13:6600-6613; Yoon *et al.* (1995) Mol. Cell. Biol. 15:2482-2489; Gonzalez and Sylvester (1995) Genomics 27:320-328; Miesfeld and Arnheim (1982) Nuc. Acids Res. 10:3933-3949); Maden *et al.* (1987) Biochem. J. 246:519-527).

- As described herein, without being bound by any theory, these specialized elements may facilitate replication and/or amplification of megabase-size chromosomal segments in the *de novo* formation of chromosomes, such as those described herein, in cells. These specialized elements are typically located in the nontranscribed intergenic spacer region upstream of the transcribed region of rDNA. The intergenic spacer region may itself contain internally repeated sequences which can be classified as tandemly repeated blocks and nontandem blocks (see e.g., Gonzalez and Sylvester (1995) Genomics 27:320-328). In mouse rDNA, an origin of bidirectional replication may be found within a 3-kb initiation zone centered approximately 1.6 kb upstream of the transcription start site (see, e.g., Gogel *et al.* (1996) Chromosoma 104:511-518). The sequences of these specialized elements tend to have an altered chromatin structure, which may be detected, for example, by nuclease hypersensitivity or the presence of AT-rich regions that can give rise to bent DNA structures. An exemplary sequence encompassing an origin of replication is shown in SEQ ID NO. 16 and in GENBANK accession no. X82564 at about positions 2430-5435.
- Exemplary sequences encompassing amplification-promoting sequences include nucleotides 690-1060 and 1105-1530 of SEQ ID NO. 16.

In human rDNA, a primary replication initiation site may be found a few kilobase pairs upstream of the transcribed region and secondary initiation sites may be found throughout the nontranscribed intergenic

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- spacer region (see, e.g., Yoon *et al.* (1995) *Mol. Cell. Biol.* **15**:2482-2489). A complete human rDNA repeat unit is presented in GENBANK as accession no. U13369 and is set forth in SEQ ID NO. 17 herein. Another exemplary sequence encompassing a replication initiation site
- 5 may be found within the sequence of nucleotides 35355-42486 in SEQ ID NO. 17 particularly within the sequence of nucleotides 37912-42486 and more particularly within the sequence of nucleotides 37912-39288 of SEQ ID NO. 17 (see Coffman *et al.* (1993) *Exp. Cell. Res.* **209**:123-132).
- 10 Cell lines containing MACs can be prepared by transforming cells, preferably a stable cell line, with a heterologous DNA fragment that encodes a selectable marker, culturing under selective conditions, and identifying cells that have a multicentric, typically dicentric, chromosome. These cells can then be manipulated as described herein to produce the
- 15 minichromosomes and other MACs, particularly the heterochromatic SATACs, as described herein.
- Development of a multicentric, particularly dicentric, chromosome typically is effected through integration of the heterologous DNA in the pericentric heterochromatin, preferably in the centromeric regions of
- 20 chromosomes carrying rDNA sequences. Thus, the frequency of incorporation can be increased by targeting to these regions, such as by including DNA, including, but not limited to, rDNA or satellite DNA, in the heterologous fragment that encodes the selectable marker. Among the preferred targeting sequences for directing the heterologous DNA to the
- 25 pericentromeric heterochromatin are rDNA sequences that target centromeric regions of chromosomes that carry rRNA genes. Such sequences include, but are not limited to, the DNA of SEQ ID NO. 16 and GENBANK accession no. X82564 and portions thereof, the DNA of SEQ ID NO. 17 and GENBANK accession no. U13369 and portions thereof,

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- and the DNA of SEQ ID NOS. 18-24. A particular vector incorporating DNA from within SEQ ID NO. 16 for use in directing integration of heterologous DNA into chromosomal rDNA is pTERPUD (see Example 12). Satellite DNA sequences can also be used to direct the
- 5 heterologous DNA to integrate into the pericentric heterochromatin. For example, vectors pTEMPUD and pHASPUD, which contain mouse and human satellite DNA, respectively, are provided herein (see Example 12) as exemplary vectors for introduction of heterologous DNA into cells for *de novo* artificial chromosome formation.
- 10 The resulting cell lines can then be treated as the exemplified cells herein to produce cells in which the dicentric chromosome has fragmented. The cells can then be used to introduce additional selective markers into the fragmented dicentric chromosome (*i.e.*, formerly dicentric chromosome), whereby amplification of the pericentric
- 15 heterochromatin will produce the heterochromatic chromosomes.
- The following discussion describes this process with reference to the EC3/7 line and the resulting cells. The same procedures can be applied to any other cells, particularly cell lines to create SATACs and euchromatic minichromosomes.
- 20 2. Formation of *de novo* chromosomes
- De novo* centromere formation in a transformed mouse LMTK-fibroblast cell line [EC3/7] after cointegration of λ constructs [λ CM8 and λ gtWESneo] carrying human and bacterial DNA [Hadlaczky *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**:8106-8110 and U.S.
- 25 application Serial No. 08/375,271] has been shown. The integration of the "heterologous" engineered human, bacterial and phage DNA, and the subsequent amplification of mouse and heterologous DNA that led to the formation of a dicentric chromosome, occurred at the centromeric region of the short arm of a mouse chromosome. By G-banding, this

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- chromosome was identified as mouse chromosome 7. Because of the presence of two functionally active centromeres on the same chromosome, regular breakages occur between the centromeres. Such specific chromosome breakages gave rise to the appearance [in
- 5 approximately 10% of the cells] of a chromosome fragment carrying the neo-centromere. From the EC3/7 cell line [see, U.S. Patent No. 5,288,625, deposited at the European Collection of Animal Cell Culture (hereinafter ECACC) under accession no. 90051001; see, also Hadlaczky et al. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**:8106-8110, and U.S.
- 10 application Serial No. 08/375,271 and the corresponding published European application EP 0 473 253, two sublines [EC3/7C5 and EC3/7C6] were selected by repeated single-cell cloning. In these cell lines, the neo-centromere was found exclusively on a minichromosome [neo-minichromosome], while the formerly dicentric chromosome carried
- 15 traces of "heterologous" DNA.

It has now been discovered that integration of DNA encoding a selectable marker in the heterochromatic region of the centromere led to formation of the dicentric chromosome.

3. The neo-minichromosome

- 20 The chromosome breakage in the EC3/7 cells, which separates the neo-centromere from the mouse chromosome, occurred in the G-band positive "heterologous" DNA region. This is supported by the observation of traces of λ and human DNA sequences at the broken end of the formerly dicentric chromosome. Comparing the G-band pattern of the
- 25 chromosome fragment carrying the neo-centromere with that of the stable neo-minichromosome, it is apparent that the neo-minichromosome is an inverted duplicate of the chromosome fragment that bears the neo-centromere. This is supported by the observation that although the neo-minichromosome carries only one functional centromere, both ends of

the minichromosome are heterochromatic, and mouse satellite DNA sequences were found in these heterochromatic regions by *in situ* hybridization.

- Mouse cells containing the minichromosome, which contains
- 5 multiple repeats of the heterologous DNA, which in the exemplified embodiment is λ DNA and the neomycin-resistance gene, can be used as recipient cells in cell transformation. Donor DNA, such as selected heterologous DNA containing λ DNA linked to a second selectable marker, such as the gene encoding hygromycin phosphotransferase
- 10 which confers hygromycin resistance [hyg], can be introduced into the mouse cells and integrated into the minichromosomes by homologous recombination of λ DNA in the donor DNA with that in the minichromosomes. Integration is verified by *in situ* hybridization and Southern blot analyses. Transcription and translation of the heterologous
- 15 DNA is confirmed by primer extension and immunoblot analyses.

For example, DNA has been targeted into the neo-minichromosome in EC3/7C5 cells using a λ DNA-containing construct [pNem1ruc] that also contains DNA encoding hygromycin resistance and the *Renilla* luciferase gene linked to a promoter, such as the cytomegalovirus [CMV]

20 early promoter, and the bacterial neomycin resistance-encoding DNA. Integration of the donor DNA into the chromosome in selected cells [designated PHN4] was confirmed by nucleic acid amplification [PCR] and *in situ* hybridization. Events that would produce a neo-minichromosome are depicted in Figure 1.

25 The resulting engineered minichromosome that contains the heterologous DNA can then be transferred by cell fusion into a recipient cell line, such as Chinese hamster ovary cells [CHO] and correct expression of the heterologous DNA can be verified. Following production of the cells, metaphase chromosomes are obtained, such as

by addition of colchicine, and the chromosomes purified by addition of AT- and GC-specific dyes on a dual laser beam based cell sorter (see Example 10 B for a description of methods of isolating artificial chromosomes). Preparative amounts of chromosomes [5 x 10⁴ - 5 x 5 10⁷ chromosomes/ml] at a purity of 95% or higher can be obtained. The resulting chromosomes are used for delivery to cells by methods such as microinjection and liposome-mediated transfer.

Thus, the neo-minichromosome is stably maintained in cells, replicates autonomously, and permits the persistent long-term expression 10 of the *neo* gene under non-selective culture conditions. It also contains megabases of heterologous known DNA [λ DNA in the exemplified embodiments] that serves as target sites for homologous recombination and integration of DNA of interest. The neo-minichromosome is, thus, a vector for genetic engineering of cells. It has been introduced into SCID 15 mice, and shown to replicate in the same manner as endogenous chromosomes.

The methods herein provide means to induce the events that lead to formation of the neo-minichromosome by introducing heterologous DNA with a selective marker [preferably a dominant selectable marker] 20 into cells and culturing the cells under selective conditions. As a result, cells that contain a multicentric, e.g., dicentric chromosome, or fragments thereof, generated by amplification are produced. Cells with the dicentric chromosome can then be treated to destabilize the chromosomes with agents, such as BrdU and/or culturing under selective 25 conditions, resulting in cells in which the dicentric chromosome has formed two chromosomes, a so-called minichromosome, and a formerly dicentric chromosome that has typically undergone amplification in the heterochromatin where the heterologous DNA has integrated to produce a SATAC or a sausage chromosome [discussed below]. These cells can

be fused with other cells to separate the minichromosome from the formerly dicentric chromosome into different cells so that each type of MAC can be manipulated separately.

4. Preparation of SATACs

5 An exemplary protocol for preparation of SATACs is illustrated in Figure 2 [particularly D, E and F] and FIGURE 3 [see, also the EXAMPLES, particularly EXAMPLES 4-7].

To prepare a SATAC, the starting materials are cells, preferably a stable cell line, such as a fibroblast cell line, and a DNA fragment that 10 includes DNA that encodes a selective marker. The DNA fragment is introduced into the cell by methods of DNA transfer, including but not limited to direct uptake using calcium phosphate, electroporation, and lipid-mediated transfer. To insure integration of the DNA fragment in the heterochromatin, it is preferable to start with DNA that will be targeted 15 to the pericentric heterochromatic region of the chromosome, such as λ CM8 and vectors provided herein, such as pTEMPUD [Figure 5] and pHASPUD (see Example 12) that include satellite DNA, or specifically into rDNA in the centromeric regions of chromosomes containing rDNA sequences. After introduction of the DNA, the cells are grown under 20 selective conditions. The resulting cells are examined and any that have multicentric, particularly dicentric, chromosomes [or heterochromatic chromosomes or sausage chromosomes or other such structure; see, Figure 2D, 2E and 2F] are selected.

In particular, if a cell with a dicentric chromosome is selected, it 25 can be grown under selective conditions, or, preferably, additional DNA encoding a second selectable marker is introduced, and the cells grown under conditions selective for the second marker. The resulting cells should include chromosomes that have structures similar to those depicted in Figures 2D, 2E, 2F. Cells with a structure, such as the

- sausage chromosome, Figure 2D, can be selected and fused with a second cell line to eliminate other chromosomes that are not of interest. If desired, cells with other chromosomes can be selected and treated as described herein. If a cell with a sausage chromosome is selected, it can
- 5 be treated with an agent, such as BrdU, that destabilizes the chromosome so that the heterochromatic arm forms a chromosome that is substantially heterochromatic [i.e., a megachromosome, see, Figure 2F]. Structures such as the gigachromosome in which the heterochromatic arm has amplified but not broken off from the
- 10 euchromatic arm, will also be observed. The megachromosome is a stable chromosome. Further manipulation, such as fusions and growth in selective conditions and/or BrdU treatment or other such treatment, can lead to fragmentation of the megachromosome to form smaller chromosomes that have the amplicon as the basic repeating unit.
- 15 The megachromosome can be further fragmented *in vivo* using a chromosome fragmentation vector, such as pTEMPUD [see, Figure 5 and EXAMPLE 12], pHASPUD or pTERPUD (see Example 12) to ultimately produce a chromosome that comprises a smaller stable replicable unit, about 15 Mb-60 Mb, containing one to four megareplicons.
- 20 Thus, the stable chromosomes formed *de novo* that originate from the short arm of mouse chromosome 7 have been analyzed. This chromosome region shows a capacity for amplification of large chromosome segments, and promotes *de novo* chromosome formation. Large-scale amplification at the same chromosome region leads to the
- 25 formation of dicentric and multicentric chromosomes, a minichromosome, the 150-200 Mb size λ neo-chromosome, the "sausage" chromosome, the 500-1000 Mb gigachromosome, and the stable 250-400 Mb megachromosome.

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A clear segmentation is observed along the arms of the megachromosome, and analyses show that the building units of this chromosome are amplicons of ~30 Mb composed of mouse major satellite DNA with the integrated "foreign" DNA sequences at both ends.

- 5 The ~30 Mb amplicons are composed of two ~15 Mb inverted doublets of ~7.5 Mb mouse major satellite DNA blocks, which are separated from each other by a narrow band of non-satellite sequences [see, e.g., Figure 3]. The wider non-satellite regions at the amplicon borders contain integrated, exogenous [heterologous] DNA, while the narrow
- 10 bands of non-satellite DNA sequences within the amplicons are integral parts of the pericentric heterochromatin of mouse chromosomes. These results indicate that the ~7.5 Mb blocks flanked by non-satellite DNA are the building units of the pericentric heterochromatin of mouse chromosomes, and the ~15 Mb size pericentric regions of mouse
- 15 chromosomes contain two ~7.5 Mb units.

Apart from the euchromatic terminal segments, the whole megachromosome is heterochromatic, and has structural homogeneity. Therefore, this large chromosome offers a unique possibility for obtaining information about the amplification process, and for analyzing some basic

- 20 characteristics of the pericentric constitutive heterochromatin, as a vector for heterologous DNA, and as a target for further fragmentation.

As shown herein, this phenomenon is generalizable and can be observed with other chromosomes. Also, although these *de novo* formed chromosome segments and chromosomes appear different, there are

- 25 similarities that indicate that a similar amplification mechanism plays a role in their formation: (i) in each case, the amplification is initiated in the centromeric region of the mouse chromosomes and large (Mb size) amplicons are formed; (ii) mouse major satellite DNA sequences are constant constituents of the amplicons, either by providing the bulk of

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the heterochromatic amplicons [H-type amplification], or by bordering the euchromatic amplicons [E-type amplification]; (iii) formation of inverted segments can be demonstrated in the λ neo-chromosome and megachromosome; (iv) chromosome arms and chromosomes formed by

- 5 the amplification are stable and functional.

The presence of inverted chromosome segments seems to be a common phenomenon in the chromosomes formed *de novo* at the centromeric region of mouse chromosome 7. During the formation of the neo-minichromosome, the event leading to the stabilization of the distal
10 segment of mouse chromosome 7 that bears the neo-centromere may have been the formation of its inverted duplicate. Amplicons of the megachromosome are inverted doublets of ~ 7.5 Mb mouse major satellite DNA blocks.

5. Cell lines

15 Cell lines that contain MACs, such as the minichromosome, the λ -neo chromosome, and the SATACs are provided herein or can be produced by the methods herein. Such cell lines provide a convenient source of these chromosomes and can be manipulated, such as by cell fusion or production of microcells for fusion with selected cell lines, to
20 deliver the chromosome of interest into hybrid cell lines. Exemplary cell lines are described herein and some have been deposited with the ECACC.

a. EC3/7C5 and EC3/7C6

Cell lines EC3/7C5 and EC3/7C6 were produced by single cell
25 cloning of EC3/7. For exemplary purposes EC3/7C5 has been deposited with the ECACC. These cell lines contain a minichromosome and the formerly dicentric chromosome from EC3/7. The stable mini-chromosomes in cell lines EC3/7C5 and EC3/7C6 appear to be the same and they seem to be duplicated derivatives of the ~ 10-15 Mb

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"broken-off" fragment of the dicentric chromosome. Their similar size in these independently generated cell lines might indicate that ~ 20-30 Mb is the minimal or close to the minimal physical size for a stable minichromosome.

5 b. **TF1004G19**

Introduction of additional heterologous DNA, including DNA encoding a second selectable marker, hygromycin phosphotransferase, i.e., the hygromycin-resistance gene, and also a detectable marker, β -galactosidase (i.e., encoded by the lacZ gene), into the EC3/7C5 cell line 10 and growth under selective conditions produced cells designated TF1004G19. In particular, this cell line was produced from the EC3/7C5 cell line by cotransfection with plasmids pH132, which contains an anti-HIV ribozyme and hygromycin-resistance gene, pCH110 [encodes β -galactosidase] and λ phage [λ cI 875 Sam 7] DNA and selection with 15 hygromycin B.

Detailed analysis of the TF1004G19 cell line by *in situ* hybridization with λ phage and plasmid DNA sequences revealed the formation of the sausage chromosome. The formerly dicentric chromosome of the EC3/7C5 cell line translocated to the end of another 20 acrocentric chromosome. The heterologous DNA integrated into the pericentric heterochromatin of the formerly dicentric chromosome and is amplified several times with megabases of mouse pericentric heterochromatic satellite DNA sequences [Fig. 2D] forming the "sausage" chromosome. Subsequently the acrocentric mouse chromosome was 25 substituted by a euchromatic telomere.

In situ hybridization with biotin-labeled subfragments of the hygromycin-resistance and β -galactosidase genes resulted in a hybridization signal only in the heterochromatic arm of the sausage

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chromosome, indicating that in TF1004G19 transformant cells these genes are localized in the pericentric heterochromatin.

A high level of gene expression, however, was detected. In general, heterochromatin has a silencing effect in Drosophila, yeast and

- 5 on the HSV-tk gene introduced into satellite DNA at the mouse centromere. Thus, it was of interest to study the TF1004G19 transformed cell line to confirm that genes located in the heterochromatin were indeed expressed, contrary to recognized dogma.

For this purpose, subclones of TF1004G19, containing a different
10 sausage chromosome [see Figure 2D], were established by single cell cloning. Southern hybridization of DNA isolated from the subclones with subfragments of hygromycin phosphotransferase and lacZ genes showed a close correlation between the intensity of hybridization and the length of the sausage chromosome. This finding supports the conclusion that
15 these genes are localized in the heterochromatic arm of the sausage chromosome.

(1) TF1004G-19C5

TF1004G-19C5 is a mouse LMTK⁻ fibroblast cell line containing neo-minichromosomes and stable "sausage" chromosomes. It is a
20 subclone of TF1004G19 and was generated by single-cell cloning of the TF1004G19 cell line. It has been deposited with the ECACC as an exemplary cell line and exemplary source of a sausage chromosome. Subsequent fusion of this cell line with CHO K20 cells and selection with hygromycin and G418 and HAT (hypoxanthine, aminopterina, and
25 thymidine medium; see Szybalski *et al.* (1962) Proc. Natl. Acad. Sci. **48**:2026) resulted in hybrid cells (designated 19C5xHa4) that carry the sausage chromosome and the neo-minichromosome. BrdU treatment of the hybrid cells, followed by single cell cloning and selection with G418

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and/or hygromycin produced various cells that carry chromosomes of interest, including GB43 and G3D5.

(2) other subclones

- Cell lines GB43 and G3D5 were obtained by treating 19C5xHa4
- 5 cells with BrdU followed by growth in G418-containing selective medium and retreatment with BrdU. The two cell lines were isolated by single cell cloning of the selected cells. GB43 cells contain the neo-minichromosome only. G3D5, which has been deposited with the ECACC, carries the neo-minichromosome and the megachromosome.
- 10 Single cell cloning of this cell line followed by growth of the subclones in G418- and hygromycin-containing medium yielded subclones such as the GHB42 cell line carrying the neo-minichromosome and the megachromosome. H1D3 is a mouse-hamster hybrid cell line carrying the megachromosome, but no neo-minichromosome, and was generated
- 15 by treating 19C5xHa4 cells with BrdU followed by growth in hygromycin-containing selective medium and single cell subcloning of selected cells. Fusion of this cell line with the CD4⁺ HeLa cell line that also carries DNA encoding an additional selection gene, the neomycin-resistance gene, produced cells [designated H1xHE41 cells] that carry the
- 20 megachromosome as well as a human chromosome that carries CD4neo. Further BrdU treatment and single cell cloning produced cell lines, such as 1B3, that include cells with a truncated megachromosome.

5. DNA constructs used to transform the cells

- Heterologous DNA can be introduced into the cells by transfection
- 25 or other suitable method at any stage during preparation of the chromosomes [see, e.g., FIG. 4]. In general, incorporation of such DNA into the MACs is assured through site-directed integration, such as may be accomplished by inclusion of λ-DNA in the heterologous DNA (for the ex amplified chromosomes), and also an additional selective marker gene.

For example, cells containing a MAC, such as the minichromosome or a SATAC, can be cotransfected with a plasmid carrying the desired heterologous DNA, such as DNA encoding an HIV ribozyme, the cystic fibrosis gene, and DNA encoding a second selectable marker, such as

5 hygromycin resistance. Selective pressure is then applied to the cells by exposing them to an agent that is harmful to cells that do not express the new selectable marker. In this manner, cells that include the heterologous DNA in the MAC are identified. Fusion with a second cell line can provide a means to produce cell lines that contain one particular

10 type of chromosomal structure or MAC.

Various vectors for this purpose are provided herein [see, Examples] and others can be readily constructed. The vectors preferably include DNA that is homologous to DNA contained within a MAC in order to target the DNA to the MAC for integration therein. The vectors also

15 include a selectable marker gene and the selected heterologous gene(s) of interest. Based on the disclosure herein and the knowledge of the skilled artisan, one of skill can construct such vectors.

Of particular interest herein is the vector pTEMPUD and derivatives thereof that can target DNA into the heterochromatic region of selected chromosomes. These vectors can also serve as fragmentation vectors

20 [see, e.g., Example 12].

Heterologous genes of interest include any gene that encodes a therapeutic product and DNA encoding gene products of interest. These genes and DNA include, but are not limited to: the cystic fibrosis gene

25 [CF], the cystic fibrosis transmembrane regulator (CFTR) gene [see, e.g., U.S. Patent No. 5,240,846; Rosenfeld *et al.* (1992) *Cell* 68:143-155; Hyde *et al.* (1993) *Nature* 362: 250-255; Kerem *et al.* (1989) *Science* 245:1073-1080; Riordan *et al.* (1989) *Science* 245:1066-1072; Rommens *et al.* (1989) *Science* 245:1059-1065; Osborne *et al.* (1991)

Am. J. Hum. Genetics 48:6089-6122; White et al. (1990) Nature 344:665-667; Dean et al. (1990) Cell 61:863-870; Erlich et al. (1991) Science 252:1643; and U.S. Patent Nos. 5,453,357, 5,449,604, 5,434,086, and 5,240,846, which provides a retroviral vector encoding 5 the normal CFTR gene].

B. Isolation of artificial chromosomes

The MACs provided herein can be isolated by any suitable method known to those of skill in the art. Also, methods are provided herein for effecting substantial purification, particularly of the SATACs. SATACs 10 have been isolated by fluorescence-activated cell sorting [FACS]. This method takes advantage of the nucleotide base content of the SATACs, which, by virtue of their high heterochromatic DNA content, will differ from any other chromosomes in a cell. In particular embodiment, metaphase chromosomes are isolated and stained with base-specific 15 dyes, such as Hoechst 33258 and chromomycin A3. Fluorescence-activated cell sorting will separate the SATACs from the endogenous chromosomes. A dual-laser cell sorter [FACS Vantage Becton Dickinson Immunocytometry Systems] in which two lasers were set to excite the dyes separately, allowed a bivariate analysis of the chromosomes by 20 base-pair composition and size. Cells containing such SATACs can be similarly sorted.

Additional methods provided herein for isolation of artificial chromosomes from endogenous chromosomes include procedures that are particularly well suited for large-scale isolation of artificial 25 chromosomes such as SATACs. In these methods, the size and density differences between SATACs and endogenous chromosomes are exploited to effect separation of these two types of chromosomes. Such methods involve techniques such as swinging bucket centrifugation, zonal rotor centrifugation, and velocity sedimentation. Affinity-,

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particularly immunoaffinity-, based methods for separation of artificial from endogenous chromosomes are also provided herein. For example, SATACs, which are predominantly heterochromatin, may be separated from endogenous chromosomes through immunoaffinity procedures

- 5 involving antibodies that specifically recognize heterochromatin, and/or the proteins associated therewith, when the endogenous chromosomes contain relatively little heterochromatin, such as in hamster cells.

C. *In vitro* construction of artificial chromosomes

Artificial chromosomes can be constructed in vitro by assembling

- 10 the structural and functional elements that contribute to a complete chromosome capable of stable replication and segregation alongside endogenous chromosomes in cells. The identification of the discrete elements that in combination yield a functional chromosome has made possible the in vitro generation of artificial chromosomes. The process of
15 in vitro construction of artificial chromosomes, which can be rigidly controlled, provides advantages that may be desired in the generation of chromosomes that, for example, are required in large amounts or that are intended for specific use in transgenic animal systems.

- For example, in vitro construction may be advantageous when
20 efficiency of time and scale are important considerations in the preparation of artificial chromosomes. Because in vitro construction methods do not involve extensive cell culture procedures, they may be utilized when the time and labor required to transform, feed, cultivate, and harvest cells used in in vivo cell-based production systems is
25 unavailable.

In vitro construction may also be rigorously controlled with respect to the exact manner in which the several elements of the desired artificial chromosome are combined and in what sequence and proportions they are assembled to yield a chromosome of precise specifications. These

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aspects may be of significance in the production of artificial chromosomes that will be used in live animals where it is desirable to be certain that only very pure and specific DNA sequences in specific amounts are being introduced into the host animal.

5 The following describes the processes involved in the construction of artificial chromosomes in vitro, utilizing a megachromosome as exemplary starting material.

1. **Identification and isolation of the components of the artificial chromosome**

10 The MACs provided herein, particularly the SATACs, are elegantly simple chromosomes for use in the identification and isolation of components to be used in the in vitro construction of artificial chromosomes. The ability to purify MACs to a very high level of purity, as described herein, facilitates their use for these purposes. For

15 example, the megachromosome, particularly truncated forms thereof [i.e. cell lines, such as 1B3 and mM2C1, which are derived from H1D3 (deposited at the European Collection of Animal Cell Culture (ECACC) under Accession No. 96040929, see EXAMPLES below) serve as starting materials.

20 For example, the mM2C1 cell line contains a micro-megachromosome (~ 50-60 kB), which advantageously contains only one centromere, two regions of integrated heterologous DNA with adjacent rDNA sequences, with the remainder of the chromosomal DNA being mouse major satellite DNA. Other truncated megachromosomes can
25 serve as a source of telomeres, or telomeres can be provided (see, Examples below regarding construction of plasmids containing tandemly repeated telomeric sequences). The centromere of the mM2C1 cell line contains mouse minor satellite DNA, which provides a useful tag for isolation of the centromeric DNA.

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Additional features of particular SATACs provided herein, such as the micro-megachromosome of the mM2C1 cell line, that make them uniquely suited to serve as starting materials in the isolation and identification of chromosomal components include the fact that the

- 5 centromeres of each megachromosome within a single specific cell line are identical. The ability to begin with a homogeneous centromere source (as opposed to a mixture of different chromosomes having differing centromeric sequences) greatly facilitates the cloning of the centromere DNA. By digesting purified megachromosomes, particularly
- 10 truncated megachromosomes, such as the micro-megachromosome, with appropriate restriction endonucleases and cloning the fragments into the commercially available and well known YAC vectors (see, e.g., Burke *et al.* (1987) *Science* **236**:806-812), BAC vectors (see, e.g., Shizuya *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**: 8794-8797 bacterial artificial
- 15 chromosomes which have a capacity of incorporating 0.9 - 1 Mb of DNA or PAC vectors (the P1 artificial chromosome vector which is a P1 plasmid derivative that has a capacity of incorporating 300 kb of DNA and that is delivered to *E. coli* host cells by electroporation rather than by bacteriophage packaging; see, e.g., Ioannou *et al.* (1994) *Nature Genetics* **6**:84-89; Pierce *et al.* (1992) *Meth. Enzymol.* **216**:549-574; Pierce *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**:2056-2060; U.S. Patent No. 5,300,431 and International PCT application No. WO 92/14819) vectors, it is possible for as few as 50 clones to represent the entire micro-megachromosome.
- 20

25 a. Centromeres

An exemplary centromere for use in the construction of a mammalian artificial chromosome is that contained within the megachromosome of any of the megachromosome-containing cell lines provided herein, such as, for example, H1D3 and derivatives thereof,

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- such as mM2C1 cells. Megachromosomes are isolated from such cell lines utilizing, for example, the procedures described herein, and the centromeric sequence is extracted from the isolated megachromosomes. For example, the megachromosomes may be separated into fragments
- 5 utilizing selected restriction endonucleases that recognize and cut at sites that, for instance, are primarily located in the replication and/or heterologous DNA integration sites and/or in the satellite DNA. Based on the sizes of the resulting fragments, certain undesired elements may be separated from the centromere-containing sequences. The centromere -
- 10 containing DNA, which could be as large as 1 Mb.

Probes that specifically recognize the centromeric sequences, such as mouse minor satellite DNA-based probes [see, e.g., Wong *et al.* (1988) *Nucl. Acids Res.* 16:11645-11661], may be used to isolate the centromere-containing YAC, BAC or PAC clones derived from the

15 megachromosome. Alternatively, or in conjunction with the direct identification of centromere-containing megachromosomal DNA, probes that specifically recognize the non-centromeric elements, such as probes specific for mouse major satellite DNA, the heterologous DNA and/or rDNA, may be used to identify and eliminate the non-centromeric DNA-

20 containing clones.

Additionally, centromere cloning methods described herein may be utilized to isolate the centromere-containing sequence of the megachromosome. For example, Example 12 describes the use of YAC vectors in combination with the murine tyrosinase gene and NMRI/Han

25 mice for identification of the centromeric sequence.

Once the centromere fragment has been isolated, it may be sequenced and the sequence information may in turn be used in PCR amplification of centromere sequences from megachromosomes or other sources of centromeres. Isolated centromeres may also be tested for

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function in vivo by transferring the DNA into a host mammalian cell. Functional analysis may include, for example, examining the ability of the centromere sequence to bind centromere-binding proteins. The cloned centromere will be transferred to mammalian cells with a selectable marker gene and the binding of a centromere-specific protein, such as anti-centromere antibodies (e.g., LU851, see, Hadlaczky *et al.* (1986) Exp. Cell Res. 167:1-15) can be used to assess function of the centromeres.

b. Telomeres

10 Preferred telomeres are the 1 kB synthetic telomere provided herein (see, Examples). A double synthetic telomere construct, which contains a 1 kB synthetic telomere linked to a dominant selectable marker gene that continues in an inverted orientation may be used for ease of manipulation. Such a double construct contains a series of 15 TTAGGG repeats 3' of the marker gene and a series of repeats of the inverted sequence, i.e., GGGATT, 5' of the marker gene as follows: (GGGATT)_n--dominant marker gene--(TTAGGG)_n. Using an inverted marker provides an easy means for insertion, such as by blunt end ligation, since only properly oriented fragments will be selected.

20 c. Megareplicator

The megareplicator sequences, such as the rDNA, provided herein are preferred for use in *in vitro* constructs. The rDNA provides an origin of replication and also provides sequences that facilitate amplification of the artificial chromosome in vivo to increase the size of the chromosome 25 to, for example accommodate increasing copies of a heterologous gene of interest as well as continuous high levels of expression of the heterologous genes.

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d. **Filler heterochromatin**

Filler heterochromatin, particularly satellite DNA, is included to maintain structural integrity and stability of the artificial chromosome and provide a structural base for carrying genes within the chromosome. The 5 satellite DNA is typically A/T-rich DNA sequence, such as mouse major satellite DNA, or G/C-rich DNA sequence, such as hamster natural satellite DNA. Sources of such DNA include any eukaryotic organisms that carry non-coding satellite DNA with sufficient A/T or G/C composition to promote ready separation by sequence, such as by FACS, 10 or by density gradients. The satellite DNA may also be synthesized by generating sequence containing monotone, tandem repeats of highly A/T- or G/C-rich DNA units.

The most suitable amount of filler heterochromatin for use in construction of the artificial chromosome may be empirically determined 15 by, for example, including segments of various lengths, increasing in size, in the construction process. Fragments that are too small to be suitable for use will not provide for a functional chromosome, which may be evaluated in cell-based expression studies, or will result in a chromosome of limited functional lifetime or mitotic and structural 20 stability.

e. **Selectable marker**

Any convenient selectable marker may be used and at any convenient locus in the MAC.

2. **Combination of the isolated chromosomal elements**

25 Once the isolated elements are obtained, they may be combined to generate the complete, functional artificial chromosome. This assembly can be accomplished for example, by in vitro ligation either in solution, LMP agarose or on microbeads. The ligation is conducted so that one end of the centromere is directly joined to a telomere. The other

end of the centromere, which serves as the gene-carrying chromosome arm, is built up from a combination of satellite DNA and rDNA sequence and may also contain a selectable marker gene. Another telomere is joined to the end of the gene-carrying chromosome arm. The gene-carrying arm is the site at which any heterologous genes of interest, for example, in expression of desired proteins encoded thereby, are incorporated either during in vitro construction of the chromosome or sometime thereafter.

3. Analysis and testing of the artificial chromosome

10 Artificial chromosomes constructed in vitro may be tested for functionality in in vivo mammalian cell systems, using any of the methods described herein for the SATACs, minichromosomes, or known to those of skill in the art.

15 **4. Introduction of desired heterologous DNA into the in vitro synthesized chromosome**

Heterologous DNA may be introduced into the in vitro synthesized chromosome using routine methods of molecular biology, may be introduced using the methods described herein for the SATACs, or may be incorporated into the in vitro synthesized chromosome as part of one 20 of the synthetic elements, such as the heterochromatin. The heterologous DNA may be linked to a selected repeated fragment, and then the resulting construct may be amplified in vitro using the methods for such in vitro amplification provided herein (see the Examples).

25 **D. Introduction of artificial chromosomes into cells, tissues, animals and plants**

Suitable hosts for introduction of the MACs provided herein, include, but are not limited to, any animal or plant, cell or tissue thereof, including, but not limited to: mammals, birds, reptiles, amphibians, insects, fish, arachnids, tobacco, tomato, wheat, plants and algae. The 30 MACs, if contained in cells, may be introduced by cell fusion or microcell

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- fusion or, if the MACs have been isolated from cells, they may be introduced into host cells by any method known to those of skill in this art, including but not limited to: direct DNA transfer, electroporation, lipid-mediated transfer, e.g., lipofection and liposomes, microprojectile
- 5 bombardment, microinjection in cells and embryos, protoplast regeneration for plants, and any other suitable method [see, e.g., Weissbach et al. (1988) *Methods for Plant Molecular Biology*, Academic Press, N.Y., Section VIII, pp. 421-463; Grierson et al. (1988) *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9; see, also U.S.
- 10 Patent Nos. 5,491,075; 5,482,928; and 5,424,409; see, also, e.g., U.S. Patent No. 5,470,708, which describes particle-mediated transformation of mammalian unattached cells].

Other methods for introducing DNA into cells include nuclear microinjection and bacterial protoplast fusion with intact cells.

- 15 Polycations, such as polybrene and polyornithine, may also be used. For various techniques for transforming mammalian cells, see e.g., Keown et al. *Methods in Enzymology* (1990) Vol. 185, pp. 527-537; and Mansour et al. (1988) *Nature* 336:348-352.

- For example, isolated, purified artificial chromosomes can be
- 20 injected into an embryonic cell line such as a human kidney primary embryonic cell line [ATCC accession number CRL 1573] or embryonic stem cells [see, e.g., Hogan et al. (1994) *Manipulating the Mouse Embryo, A :Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, see, especially, pages 255-264 and
- 25 Appendix 3].

Preferably the chromosomes are introduced by microinjection, using a system such as the Eppendorf automated microinjection system, and grown under selective conditions, such as in the presence of hygromycin B or neomycin.

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1. Methods for introduction of chromosomes into hosts

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. These methods include any, including those described herein, known to those of skill in the art.

5 a. DNA uptake

For mammalian cells that do not have cell walls, the calcium phosphate precipitation method for introduction of exogenous DNA [see, e.g., Graham *et al.* (1978) *Virology* **52**:456-457; Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**:1373-1376; and *Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)] is often preferred. DNA uptake can be accomplished by DNA alone or in the presence of polyethylene glycol [PEG-mediated gene transfer], which is a fusion agent, or by any variations of such methods known to those of skill in the art [see, e.g., U.S. Pat. No. 15 4,684,611].

Lipid-mediated carrier systems are also among the preferred methods for introduction of DNA into cells [see, e.g., Teifel *et al.* (1995) *Biotechniques* **19**:79-80; Albrecht *et al.* (1996) *Ann. Hematol.* **72**:73-79; Holmen *et al.* (1995) *In Vitro Cell Dev. Biol. Anim.* **31**:347-351; Remy *et al.* (1994) *Bioconjug. Chem.* **5**:647-654; Le Bolc'h *et al.* (1995) *Tetrahedron Lett.* **36**:6681-6684; Loeffler *et al.* (1993) *Meth. Enzymol.* **217**:599-618]. Lipofection [see, e.g., Strauss (1996) *Meth. Mol. Biol.* **54**:307-327] may also be used to introduce DNA into cells. This method is particularly well-suited for transfer of exogenous DNA into chicken cells (e.g., chicken blastodermal cells and primary chicken fibroblasts; see Brazolot *et al.* (1991) *Mol. Repro. Dev.* **30**:304-312). In particular, DNA of interest can be introduced into chickens in operative linkage with promoters from genes, such as lysozyme and ovalbumin, that are

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expressed in the egg, thereby permitting expression of the heterologous DNA in the egg.

Additional methods useful in the direct transfer of DNA into cells include particle gun electrofusion [see, e.g., U.S. Patent Nos. 4,955,378, 5 4,923,814, 4,476,004, 4,906,576 and 4,441,972] and virion-mediated gene transfer.

A commonly used approach for gene transfer in land plants involves the direct introduction of purified DNA into protoplasts. The three basic methods for direct gene transfer into plant cells include: 1) polyethylene 10 glycol [PEG]-mediated DNA uptake, 2) electroporation-mediated DNA uptake and 3) microinjection. In addition, plants may be transformed using ultrasound treatment [see, e.g., International PCT application publication No. WO 91/00358].

b. Electroporation

15 Electroporation involves providing high-voltage electrical pulses to a solution containing a mixture of protoplasts and foreign DNA to create reversible pores in the membranes of plant protoplasts as well as other cells. Electroporation is generally used for prokaryotes or other cells, such as plants that contain substantial cell-wall barriers. Methods for 20 effecting electroporation are well known [see, e.g., U.S. Patent Nos. 4,784,737, 5,501,967, 5,501,662, 5,019,034, 5,503,999; see, also Frommet al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82:5824-5828].

For example, electroporation is often used for transformation of plants [see, e.g., Ag Biotechnology News 7:3 and 17 25 (September/October 1990)]. In this technique, plant protoplasts are electroporated in the presence of the DNA of interest that also includes a phenotypic marker. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form

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plant callus. Transformed plant cells will be identified by virtue of the expressed phenotypic marker. The exogenous DNA may be added to the protoplasts in any form such as, for example, naked linear, circular or supercoiled DNA, DNA encapsulated in liposomes, DNA in spheroplasts,

- 5 DNA in other plant protoplasts, DNA complexed with salts, and other methods.

c. Microcells

The chromosomes can be transferred by preparing microcells containing an artificial chromosome and then fusing with selected target

- 10 cells. Methods for such preparation and fusion of microcells are well known [see the Examples and also see, e.g., U.S. Patent Nos.

5,240,840, 4,806,476, 5,298,429, 5,396,767, Fournier (1981) Proc. Natl. Acad. Sci. U.S.A. 78:6349-6353; and Lambert et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88:5907-59]. Microcell fusion, using microcells

- 15 that contain an artificial chromosome, is a particularly useful method for introduction of MACs into avian cells, such as DT40 chicken pre-B cells [for a description of DT40 cell fusion, see, e.g., Dieken et al. (1996) Nature Genet. 12:174-182].

2. Hosts

- 20 Suitable hosts include any host known to be useful for introduction and expression of heterologous DNA. Of particular interest herein, animal and plant cells and tissues, including, but not limited to insect cells and larvae, plants, and animals, particularly transgenic (non-human) animals, and animal cells. Other hosts include, but are not limited to
- 25 mammals, birds, particularly fowl such as chickens, reptiles, amphibians, insects, fish, arachnids, tobacco, tomato, wheat, monocots, dicots and algae, and any host into which introduction of heterologous DNA is

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desired. Such introduction can be effected using the MACs provided herein, or, if necessary by using the MACs provided herein to identify species-specific centromeres and/or functional chromosomal units and then using the resulting centromeres or chromosomal units as artificial

5 chromosomes, or alternatively, using the methods exemplified herein for production of MACs to produce species-specific artificial chromosomes.

a. **Introduction of DNA into embryos for production of transgenic (non-human) animals and introduction of DNA into animal cells**

10 Transgenic (non-human) animals can be produced by introducing exogenous genetic material into a pronucleus of a mammalian zygote by microinjection [see, e.g., U.S. Patent Nos. 4,873,191 and 5,354,674; see, also, International PCT application publication No. WO 95/14769, which is based on U.S. application Serial No. 08/159,084]. The zygote
15 is capable of development into a mammal. The embryo or zygote is transplanted into a host female uterus and allowed to develop. Detailed protocols and examples are set forth below.

Nuclear transfer [see, Wilmut *et al.* (1997) Nature 385:810-813, International PCT application Nos. WO 97/07669 and WO 97/07668].

20 Briefly in this method, the SATAC containing the genes of interest is introduced by any suitable method, into an appropriate donor cell, such as a mammary gland cell, that contains totipotent nuclei. The diploid nucleus of the cell, which is either in G0 or G1 phase, is then introduced, such as by cell fusion or microinjection, into an unactivated oocyte,
25 preferably enucleated cell, which is arrested in the metaphase of the second meiotic division. Enucleation may be effected by any suitable method, such as actual removal, or by treating with means, such as ultraviolet light, that functionally remove the nucleus. The oocyte is then activated, preferably after a period of contact, about 6-20 hours for
30 cattle, of the new nucleus with the cytoplasm, while maintaining correct

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ploidy, to produce a reconstituted embryo, which is then introduced into a host. Ploidy is maintained during activation, for example, by incubating the reconstituted cell in the presence of a microtubule inhibitor, such as nocodazole, colchicine, cocemid, and taxol, whereby the DNA replicates

5 once.

Transgenic chickens can be produced by injection of dispersed blastodermal cells from Stage X chicken embryos into recipient embryos at a similar stage of development [see e.g., Etches et al. (1993) Poultry Sci. 72:882-889; Petitte et al. (1990) Development 108:185-189].

10 Heterologous DNA is first introduced into the donor blastodermal cells using methods such as, for example, lipofection [see, e.g., Brazolot et al. (1991) Mol. Repro. Dev. 30:304-312] or microcell fusion [see, e.g., Dieken et al. (1996) Nature Genet. 12:174-182]. The transfected donor cells are then injected into recipient chicken embryos [see e.g., Carsience
15 et al. (1993) Development 117: 669-675]. The recipient chicken embryos within the shell are candled and allowed to hatch to yield a germline chimeric chicken.

DNA can be introduced into animal cells using any known procedure, including, but not limited to: direct uptake, incubation with
20 polyethylene glycol [PEG], microinjection, electroporation, lipofection, cell fusion, microcell fusion, particle bombardment, including microprojectile bombardment [see, e.g., U.S. Patent No. 5,470,708, which provides a method for transforming unattached mammalian cells via particle bombardment], and any other such method. For example, the transfer of
25 plasmid DNA in liposomes directly to human cells *in situ* has been approved by the FDA for use in humans [see, e.g., Nabel, et al. (1990) Science 249:1285-1288 and U.S. Patent No. 5,461,032].

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b. Introduction of heterologous DNA into plants

Numerous methods for producing or developing transgenic plants are available to those of skill in the art. The method used is primarily a function of the species of plant. These methods include, but are not limited to: direct transfer of DNA by processes, such as PEG-induced DNA uptake, protoplast fusion, microinjection, electroporation, and microparticle bombardment [see, e.g., Uchimiya *et al.* (1989) *J. of Biotech.* 12: 1-20 for a review of such procedures, see, also, e.g., U.S. Patent Nos. 5,436,392 and 5,489,520 and many others]. For purposes herein, when introducing a MAC, microinjection, protoplast fusion and particle gun bombardment are preferred.

Plant species, including tobacco, rice, maize, rye, soybean, Brassica napus, cotton, lettuce, potato and tomato, have been used to produce transgenic plants. Tobacco and other species, such as petunias, often serve as experimental models in which the methods have been developed and the genes first introduced and expressed.

DNA uptake can be accomplished by DNA alone or in the presence of PEG, which is a fusion agent, with plant protoplasts or by any variations of such methods known to those of skill in the art [see, e.g., U.S. Patent No. 4,684,611 to Schilperoort *et al.*]. Electroporation, which involves high-voltage electrical pulses to a solution containing a mixture of protoplasts and foreign DNA to create reversible pores, has been used, for example, to successfully introduce foreign genes into rice and Brassica napus. Microinjection of DNA into plant cells, including cultured cells and cells in intact plant organs and embryoids in tissue culture and microparticle bombardment [acceleration of small high density particles, which contain the DNA, to high velocity with a particle gun apparatus, which forces the particles to penetrate plant cell walls and membranes] have also been used. All plant cells into which DNA can be introduced

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and that can be regenerated from the transformed cells can be used to produce transformed whole plants which contain the transferred artificial chromosome. The particular protocol and means for introduction of the DNA into the plant host may need to be adapted or refined to suit the 5 particular plant species or cultivar.

c. Insect cells

Insects are useful hosts for introduction of artificial chromosomes for numerous reasons, including, but not limited to: (a) amplification of genes encoding useful proteins can be accomplished in the artificial 10 chromosome to obtain higher protein yields in insect cells; (b) insect cells support required post-translational modifications, such as glycosylation and phosphorylation, that can be required for protein biological functioning; (c) insect cells do not support mammalian viruses, and, thus, eliminate the problem of cross-contamination of products with such 15 infectious agents; (d) this technology circumvents traditional recombinant baculovirus systems for production of nutritional, industrial or medicinal proteins in insect cell systems; (e) the low temperature optimum for insect cell growth (28° C) permits reduced energy cost of production; (f) serum-free growth medium for insect cells permits lower production 20 costs; (g) artificial chromosome-containing cells can be stored indefinitely at low temperature; and (h) insect larvae will be biological factories for production of nutritional, medicinal or industrial proteins by microinjection of fertilized insect eggs [see, e.g., Joy *et al.* (1991) Current Science 66:145-150, which provides a method for microinjecting heterologous 25 DNA into *Bombyx mori* eggs].

Either MACs or insect-specific artificial chromosomes [BUGACs] will be used to introduce genes into insects. As described in the Examples, it appears that MACs will function in insects to direct expression of heterologous DNA contained thereon. For example, as

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- described in the Examples, a MAC containing the *B. mori* actin gene promoter fused to the lacZ gene has been generated by transfection of EC3/7C5 cells with a plasmid containing the fusion gene. Subsequent fusion of the *B. mori* cells with the transfected EC3/7C5 cells that
- 5 survived selection yielded a MAC-containing insect-mouse hybrid cell line in which β -galactosidase expression was detectable.

Insect host cells include, but are not limited to, hosts such as *Spodoptera frugiperda* [caterpillar], *Aedes aegypti* [mosquito], *Aedes albopictus* [mosquito], *Drosophila melanogaster* [fruitfly], *Bombyx mori* [silkworm], *Manduca sexta* [tomato horn worm] and *Trichoplusia ni* [cabbage looper]. Efforts have been directed toward propagation of insect cells in culture. Such efforts have focused on the fall armyworm, *Spodoptera frugiperda*. Cell lines have been developed also from other insects such as the cabbage looper, *Trichoplusia ni* and the silkworm,

10 *Bombyx mori*. It has also been suggested that analogous cell lines can be created using the tomato hornworm, *Manduca sexta*. To introduce DNA into an insect, it should be introduced into the larvae, and allowed to proliferate, and then the hemolymph recovered from the larvae so that the proteins can be isolated therefrom.

15

20 The preferred method herein for introduction of artificial chromosomes into insect cells is microinjection [see, e.g., Tamura et al. (1991) *Bio Ind.* 8:26-31; Nikolaev et al. (1989) *Mol. Biol. (Moscow)* 23:1177-87; and methods exemplified and discussed herein].

E. Applications for and Uses of Artificial chromosomes

25 Artificial chromosomes provide convenient and useful vectors, and in some instances [e.g., in the case of very large heterologous genes] the only vectors, for introduction of heterologous genes into hosts. Virtually any gene of interest is amenable to introduction into a host via artificial chromosomes. Such genes include, but are not limited to, genes that

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encode receptors, cytokines, enzymes, proteases, hormones, growth factors, antibodies, tumor suppressor genes, therapeutic products and multigene pathways.

The artificial chromosomes provided herein will be used in methods 5 of protein and gene product production, particularly using insects as host cells for production of such products, and in cellular (e.g., mammalian cell) production systems in which the artificial chromosomes (particularly MACs) provide a reliable, stable and efficient means for optimizing the biomanufacturing of important compounds for medicine 10 and industry. They are also intended for use in methods of gene therapy, and for production of transgenic plants and animals [discussed above, below and in the EXAMPLES].

1. Gene Therapy

Any nucleic acid encoding a therapeutic gene product or product 15 of a multigene pathway may be introduced into a host animal, such as a human, or into a target cell line for introduction into an animal, for therapeutic purposes. Such therapeutic purposes include, genetic therapy to cure or to provide gene products that are missing or defective, to deliver agents, such as anti-tumor agents, to targeted cells or to an 20 animal, and to provide gene products that will confer resistance or reduce susceptibility to a pathogen or ameliorate symptoms of a disease or disorder. The following are some exemplary genes and gene products. Such exemplification is not intended to be limiting.

a. Anti-HIV ribozymes

25 As exemplified below, DNA encoding anti-HIV ribozymes can be introduced and expressed in cells using MACs, including the euchromatin-based minichromosomes and the SATACs. These MACs can be used to make a transgenic mouse that expresses a ribozyme and, thus, serves as a model for testing the activity of such ribozymes or from

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which ribozyme-producing cell lines can be made. Also, introduction of a MAC that encodes an anti-HIV ribozyme into human cells will serve as treatment for HIV infection. Such systems further demonstrate the viability of using any disease-specific ribozyme to treat or ameliorate a
5 particular disease.

b. Tumor Suppressor Genes

Tumor suppressor genes are genes that, in their wild-type alleles, express proteins that suppress abnormal cellular proliferation. When the gene coding for a tumor suppressor protein is mutated or deleted, the
10 resulting mutant protein or the complete lack of tumor suppressor prot in expression may result in a failure to correctly regulate cellular proliferation. Consequently, abnormal cellular proliferation may take place, particularly if there is already existing damage to the cellular regulatory mechanism. A number of well-studied human tumors and
15 tumor cell lines have been shown to have missing or nonfunctional tumor suppressor genes.

Examples of tumor suppression genes include, but are not limited to, the retinoblastoma susceptibility gene or RB gene, the p53 gene, the gene that is deleted in colon carcinoma [i.e., the DCC gene] and the
20 neurofibromatosis type 1 [NF-1] tumor suppressor gene [see, e.g., U.S. Patent No. 5,496,731; Weinberg *et al.* (1991) 254:1138-1146]. Loss of function or inactivation of tumor suppressor genes may play a central role in the initiation and/or progression of a significant number of human cancers.

25 The p53 Gene

Somatic cell mutations of the p53 gene are said to be the most frequent of the gene mutations associated with human cancer [s e, e.g., Weinberg *et al.* (1991) Science 254:1138-1146]. The normal or wild-type p53 gene is a negative regulator of cell growth, which, when

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damaged, favors cell transformation. The p53 expression product is found in the nucleus, where it may act in parallel or cooperatively with other gene products. Tumor cell lines in which p53 has been deleted have been successfully treated with wild-type p53 vector to reduce

5 tumorigenicity [see, Baker *et al.* (1990) Science **249**:912-915].

DNA encoding the p53 gene and plasmids containing this DNA are well known [see, e.g., U.S. Patent No. 5,260,191; see, also Chen *et al.* (1990) Science **250**:1576; Farrel *et al.* (1991) EMBO J. **10**:2879-2887; plasmids containing the gene are available from the ATCC, and the 10 sequence is in the GenBank Database, accession nos. X54156, X60020, M14695, M16494, K03199].

c. The CFTR gene

Cystic fibrosis [CF] is an autosomal recessive disease that affects epithelia of the airways, sweat glands, pancreas, and other organs. It is 15 a lethal genetic disease associated with a defect in chloride ion transport, and is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator [CFTR], a 1480 amino acid protein that has been associated with the expression of chloride conductance in a variety of eukaryotic cell types. Defects in CFTR destroy or reduce the 20 ability of epithelial cells in the airways, sweat glands, pancreas and other tissues to transport chloride ions in response to cAMP-mediated agonists and impair activation of apical membrane channels by cAMP-dependent protein kinase A [PKA]. Given the high incidence and devastating nature of this disease, development of effective CF treatments is imperative.

25 The CFTR gene (~ 250 kb) can be transferred into a MAC for use, for example, in gene therapy as follows. A CF-YAC [see Green *et al.* Science **250**:94-98] may be modified to include a selectable marker, such as a gene encoding a protein that confers resistance to puromycin or hygromycin, and λ-DNA for use in site-specific integration into a neo-

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minichromosome or a SATAC. Such a modified CF-YAC can be introduced into MAC-containing cells, such as EC3/7C5 or 19C5xHa4 cells, by fusion with yeast protoplasts harboring the modified CF-YAC or microinjection of yeast nuclei harboring the modified CF-YAC into the 5 cells. Stable transformants are then selected on the basis of antibiotic resistance. These transformants will carry the modified CF-YAC within the MAC contained in the cells.

2. Animals, birds, fish and plants that are genetically altered to possess desired traits such as resistance to disease

10 Artificial chromosomes are ideally suited for preparing animals, including vertebrates and invertebrates, including birds and fish as well as mammals, that possess certain desired traits, such as, for example, disease resistance, resistance to harsh environmental conditions, altered growth patterns, and enhanced physical characteristics.

15 One example of the use of artificial chromosomes in generating disease-resistant organisms involves the preparation of multivalent vaccines. Such vaccines include genes encoding multiple antigens that can be carried in a MAC, or species-specific artificial chromosome, and either delivered to a host to induce immunity, or incorporated into 20 embryos to produce transgenic (non-human) animals and plants that are immune or less susceptible to certain diseases.

Disease-resistant animals and plants may also be prepared in which resistance or decreased susceptibility to disease is conferred by introduction into the host organism or embryo of artificial chromosomes 25 containing DNA encoding gene products (e.g., ribozymes and proteins that are toxic to certain pathogens) that destroy or attenuate pathogens or limit access of pathogens to the host.

Animals and plants possessing desired traits that might, for example, enhance utility, processibility and commercial value of the

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organisms in areas such as the agricultural and ornamental plant industries may also be generated using artificial chromosomes in the same manner as described above for production of disease-resistant animals and plants. In such instances, the artificial chromosomes that 5 are introduced into the organism or embryo contain DNA encoding gene products that serve to confer the desired trait in the organism.

Birds, particularly fowl such as chickens, fish and crustaceans will serve as model hosts for production of genetically altered organisms using artificial chromosomes.

10 3. **Use of MACs and other artificial chromosomes for preparation and screening of libraries**

Since large fragments of DNA can be incorporated into each artificial chromosome, the chromosomes are well-suited for use as cloning vehicles that can accommodate entire genomes in the preparation 15 of genomic DNA libraries, which then can be readily screened. For example, MACs may be used to prepare a genomic DNA library useful in the identification and isolation of functional centromeric DNA from different species of organisms. In such applications, the MAC used to prepare a genomic DNA library from a particular organism is one that is 20 not functional in cells of that organism. That is, the MAC does not stably replicate, segregate or provide for expression of genes contained within it in cells of the organism. Preferably, the MACs contain an indicator gene (e.g., the lacZ gene encoding β -galactosidase or genes encoding products that confer resistance to antibiotics such as 25 neomycin, puromycin, hygromycin) linked to a promoter that is capable of promoting transcription of the indicator gene in cells of the organism. Fragments of genomic DNA from the organism are incorporated into the MACs, and the MACs are transferred to cells from the organism. Cells that contain MACs that have incorporated functional centromeres

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contained within the genomic DNA fragments are identified by detection of expression of the marker gene.

4. **Use of MACs and other artificial chromosomes for stable, high-level protein production**

- 5 Cells containing the MACs and/or other artificial chromosomes provided herein are advantageously used for production of proteins, particularly several proteins from one cell line, such as multiple proteins involved in a biochemical pathway or multivalent vaccines. The genes encoding the proteins are introduced into the artificial chromosomes
- 10 which are then introduced into cells. Alternatively, the heterologous gene(s) of interest are transferred into a production cell line that already contains artificial chromosomes in a manner that targets the gene(s) to the artificial chromosomes. The cells are cultured under conditions whereby the heterologous proteins are expressed. Because the proteins
- 15 will be expressed at high levels in a stable permanent extra-genomic chromosomal system, selective conditions are not required.

Any transfectable cells capable of serving as recombinant hosts adaptable to continuous propagation in a cell culture system [see, e.g., McLean (1993) Trends In Biotech. 11:232-238] are suitable for use in an

20 artificial chromosome-based protein production system. Exemplary host cell lines include, but are not limited to, the following: Chinese hamster ovary (CHO) cells [see, e.g., Zang *et al.* (1995) Biotechnology 13:389-392], HEK 293, Ltk⁻, COS-7, DG44, and BHK cells. CHO cells are particularly preferred host cells. Selection of host cell lines for use in

25 artificial chromosome-based protein production systems is within the skill of the art, but often will depend on a variety of factors, including the properties of the heterologous protein to be produced, potential toxicity of the protein in the host cell, any requirements for post-translational modification (e.g., glycosylation, amination, phosphorylation) of the

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protein, transcription factors available in the cells, the type of promoter element(s) being used to drive expression of the heterologous gene, whether production will be completely intracellular or the heterologous protein will preferably be secreted from the cell, and the types of

- 5 processing enzymes in the cell.

The artificial chromosome-based system for heterologous protein production has many advantageous features. For example, as described above, because the heterologous DNA is located in an independent, extra-genomic artificial chromosome (as opposed to randomly inserted in

- 10 an unknown area of the host cell genome or located as extrachromosomal element(s) providing only transient expression) it is stably maintained in an active transcription unit and is not subject to ejection via recombination or elimination during cell division.

Accordingly, it is unnecessary to include a selection gene in the host

- 15 cells and thus growth under selective conditions is also unnecessary. Furthermore, because the artificial chromosomes are capable of incorporating large segments of DNA, multiple copies of the heterologous gene and linked promoter element(s) can be retained in the chromosomes, thereby providing for high-level expression of the foreign

- 20 protein(s). Alternatively, multiple copies of the gene can be linked to a single promoter element and several different genes may be linked in a fused polygene complex to a single promoter for expression of, for example, all the key proteins constituting a complete metabolic pathway [see, e.g., Beck von Bodman *et al.* (1995) *Biotechnology* 13:587-591].

- 25 Alternatively, multiple copies of a single gene can be operatively linked to a single promoter, or each or one or several copies may be linked to different promoters or multiple copies of the same promoter.

Additionally, because artificial chromosomes have an almost unlimited capacity for integration and expression of foreign genes, they can be

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- used not only for the expression of genes encoding end-products of interest, but also for the expression of genes associated with optimal maintenance and metabolic management of the host cell, e.g., genes encoding growth factors, as well as genes that may facilitate rapid
- 5 synthesis of correct form of the desired heterologous protein product, e.g., genes encoding processing enzymes and transcription factors. The MACS are suitable for expression of any proteins or peptides, including proteins and peptides that require in vivo posttranslational modification for their biological activity. Such proteins include, but are
- 10 not limited to antibody fragments, full-length antibodies, and multimeric antibodies, tumor suppressor proteins, naturally occurring or artificial antibodies and enzymes, heat shock proteins, and others.

Thus, such cell-based "protein factories" employing MACs can be generated using MACs constructed with multiple copies [theoretically an

15 unlimited number or at least up to a number such that the resulting MAC is about up to the size of a genomic chromosome (i.e., endogenous)] of protein-encoding genes with appropriate promoters, or multiple genes driven by a single promoter, i.e., a fused gene complex [such as a complete metabolic pathway in plant expression system; see, e.g., Beck

20 von Bodman (1995) Biotechnology 13:587-591]. Once such MAC is constructed, it can be transferred to a suitable cell culture system, such as a CHO cell line in protein-free culture medium [see, e.g., (1995) Biotechnology 13:389-39] or other immortalized cell lines [see, e.g., (1993) TIBTECH 11:232-238] where continuous production can be

25 established.

The ability of MACs to provide for high-level expression of heterologous proteins in host cells is demonstrated, for example, by analysis of the H1D3 and G3D5 cell lines described herein and deposited with the ECACC. Northern blot analysis of mRNA obtained from these

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cells reveals that expression of the hygromycin-resistance and β -galactosidase genes in the cells correlates with the amplicon number of the megachromosome(s) contained therein.

5 F. Methods for the synthesis of DNA sequences containing repeated DNA units

Generally, assembly of tandemly repeated DNA poses difficulties such as unambiguous annealing of the complementary oligos. For example, separately annealed products may ligate in an inverted 10 orientation. Additionally, tandem or inverted repeats are particularly susceptible to recombination and deletion events that may disrupt the sequence. Selection of appropriate host organisms (e.g., rec strains) for use in the cloning steps of the synthesis of sequences of tandemly repeated DNA units may aid in reduction and elimination of such events.

15 Methods are provided herein for the synthesis of extended DNA sequences containing repeated DNA units. These methods are particularly applicable to the synthesis of arrays of tandemly repeated DNA units, which are generally difficult or not possible to construct utilizing other known gene assembly strategies. A specific use of these 20 methods is in the synthesis of sequences of any length containing simple (e.g., ranging from 2-6 nucleotides) tandem repeats (such as telomeres and satellite DNA repeats and trinucleotide repeats of possible clinical significance) as well as complex repeated DNA sequences. An particular example of the synthesis of a telomere sequence containing over 150 25 successive repeated hexamers utilizing these methods is provided herein.

The methods provided herein for synthesis of arrays of tandem DNA repeats are based in a series of extension steps in which successive doublings of a sequence of repeats results in an exponential expansion of the array of tandem repeats. These methods provide several advantages 30 over previously known methods of gene assembly. For instance, the

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starting oligonucleotides are used only once. The intermediates in, as well as the final product of, the construction of the DNA arrays described herein may be obtained in cloned form in a microbial organism (e.g., E. coli and yeast). Of particular significance, with regard to these methods

- 5 is the fact that sequence length increases exponentially, as opposed to linearly, in each extension step of the procedure even though only two oligonucleotides are required in the methods. The construction process does not depend on the compatibility of restriction enzyme recognition sequences and the sequence of the repeated DNA because restriction

10 sites are used only temporarily during the assembly procedure. No adaptor is necessary, though a region of similar function is located between two of the restriction sites employed in the process. The only limitation with respect to restriction site use is that the two sites employed in the method must not be present elsewhere in the vector

15 utilized in any cloning steps. These procedures can also be used to construct complex repeats with perfectly identical repeat units, such as the variable number tandem repeat (VNTR) 3' of the human apolipoprotein B100 gene (a repeat unit of 30 bp, 100% AT) or alphoid satellite DNA.

20 The method of synthesizing DNA sequences containing tandem repeats may generally be described as follows.

1. Starting materials

Two oligonucleotides are utilized as starting materials.

- Oligonucleotide 1 is of length k of repeated sequence (the flanks of
which are not relevant) and contains a relatively short stretch (60-90
nucleotides) of the repeated sequence, flanked with appropriately chosen
restriction sites:

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wherein S1 is restriction site 1 cleaved by E1 [preferably an enzyme producing a 3'-overhang (e.g., PacI, PstI, SphI, NsiI, etc.) or blunt-end], S2 is a second restriction site cleaved by E2 (preferably an enzyme producing a 3'-overhang or one that cleaves outside the recognition sequence, such as TspRI), > represents a simple repeat unit, and '—' denotes a short (8-10) nucleotide flanking sequence complementary to oligonucleotide 2:

5 3'- — S3-5'

10 wherein S3 is a third restriction site for enzyme E3 and which is present in the vector to be used during the construction.

Because there is a large variety of restriction enzymes that recognize many different DNA sequences as cleavage sites, it should always be possible to select sites and enzymes (preferably those that yield a 3'-protruding end) suitable for these methods in connection with the synthesis of any one particular repeat array. In most cases, only 1 (or perhaps 2) nucleotide(s) has of a restriction site is required to be present in the repeat sequence, and the remaining nucleotides of the restriction site can be removed, for example:

15 PacI: TTAAT/TAA-- (Klenow/dNTP) TAA--

20 PstI: CTGCA/G-- (Klenow/dNTP) G--

NsiI: ATGCA/T-- (Klenow/dNTP) T--

KpnI: GGTAC/C-- (Klenow/dNTP) C--

Though there is no known restriction enzyme leaving a single A behind, this problem can be solved with enzymes leaving behind none at all, for example:

25 TaiI: ACGT/ (Klenow/dNTP) --

NlaIII: CATG/ (Klenow/dNTP) --

Additionally, if mung bean nuclease is used instead of Klenow, then the following

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XbaI: T/CTAGA Mung bean nuclease A-

Furthermore, there are a number of restriction enzymes that cut outside of the recognition sequence, and in this case, there is no limitation at all:

- 5 TspRI NNCAGTGNN/- (Klenow/dNTP) --
BsmI GAATG CN/- (Klenow/dNTP) --
CTTAC/GN -- (Klenow/dNTP) --

2. Step 1 - Annealing

Oligonucleotides 1 and 2 are annealed at a temperature selected

- 10 depending on the length of overlap (typically in the range of 30-65 °C).

3. Step 2 - Generating a double-stranded molecule

The annealed oligonucleotides are filled-in with Klenow polymerase in the presence of dNTP to produce a double-stranded (ds) sequence:

4. Step 3 - Incorporation of double-stranded DNA into a vector

The double-stranded DNA is cleaved with restriction enzymes E1 and E3 and subsequently ligated into a vector (e.g., pUC19 or a yeast vector) that has been cleaved with the same enzymes E1 and E3. The

- 20 ligation product is used to transform competent host cells compatible with the vector being used (e.g., when pUC19 is used, bacterial cells such as E. coli DH5 α are suitable hosts) which are then plated onto selection plates. Recombinants can be identified either by color (e.g., by X-gal staining for β -galactosidase expression) or by colony hybridization

25 using ^{32}P -labeled oligonucleotide 2 (detection by hybridization to oligonucleotide 2 is preferred because its sequence is removed in each of the subsequent extension steps and thus is present only in recombinants that contain DNA that has undergone successful extension of the repeated sequence).

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5. Step 4 - Isolation of insert from the plasmid

An aliquot of the recombinant plasmid containing k nucleotides of the repeat sequence is digested with restriction enzymes E1 and E3, and the insert is isolated on a gel (native polyacrylamide while the insert is

- 5 short, but agarose can be used for isolation of longer inserts in subsequent steps). A second aliquot of the recombinant plasmid is cut with enzymes E2 (treated with Klenow and dNTP to remove the 3'-overhang) and E3, and the large fragment (plasmid DNA plus the insert) is isolated.

10 **6. Step 5 - Extension of the DNA sequence of k repeats**

- The two DNAs (the S1-S3 insert fragment and the vector plus insert) are ligated, plated to selective plates, and screened for extended recombinants as in Step 3. Now the length of the repeat sequence between restriction sites is twice that of the repeat sequence in the
- 15 previous step, i.e., $2k$.

7. Step 6 - Extension of the DNA sequence of $2k$ repeats

- Steps 4 and 5 are repeated as many times as needed to achieve the desired repeat sequence size. In each extension cycle, the repeat sequence size doubles, i.e., if m is the number of extension cycles, the
- 20 size of the repeat sequence will be $k \times 2^m$ nucleotides.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

General Materials and Methods

- 25 The following materials and methods are exemplary of methods that are used in the following Examples and that can be used to prepare cell lines containing artificial chromosomes. Other suitable materials and methods known to those of skill in the art may be used. Modifications of

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these materials and methods known to those of skill in the art may also be employed.

A. Culture of cell lines, cell fusion, and transfection of cells

1. Chinese hamster K-20 cells and mouse A9 fibroblast
5 cells were cultured in F-12 medium. EC3/7 [see, U.S. Patent No. 5,288,625, and deposited at the European Collection of Animal cell Culture (ECACC) under accession no. 90051001; see, also Hadlaczky et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88:8106-8110 and U.S. application Serial No. 08/375,271] and EC3/7C5 [see, U.S. Patent No. 10 5,288,625 and Praznovszky et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88:11042-11046] mouse cell lines, and the KE1-2/4 hybrid cell line were maintained in F-12 medium containing 400 µg/ml G418 [SIGMA, St. Louis, MO].

2. TF1004G19 and TF1004G-19C5 mouse cells,
15 described below, and the 19C5xHa4 hybrid, described below, and its sublines were cultured in F-12 medium containing up to 400 µg/ml Hygromycin B [Calbiochem]. LP11 cells were maintained in F-12 medium containing 3-15 µg/ml Puromycin [SIGMA, St. Louis, MO].

3. Cotransfection of EC3/7C5 cells with plasmids
20 [pH132, pCH110 available from Pharmacia, see, also Hall et al. (1983) J. Mol. Appl. Gen. 2:101-109] and with λ DNA was conducted using the calcium phosphate DNA precipitation method [see, e.g., Chen et al. (1987) Mol. Cell. Biol. 7:2745-2752], using 2-5 µg plasmid DNA and 25 20 µg λ phage DNA per 5×10^6 recipient cells.

4. Cell fusion

Mouse and hamster cells were fused using polyethylene glycol [Davidson et al. (1976) Som. Cell Genet. 2:165-176]. Hybrid cells were selected in HAT medium containing 400 µg/ml Hygromycin B.

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Approximately 2×10^7 recipient and 2×10^6 donor cells were fused using polyethylene glycol [Davidson *et al.* (1976) Som. Cell Genet. 2:165-176]. Hybrids were selected and maintained in F-12/HAT medium [Szybalsky *et al.* (1962) Natl. Cancer Inst. Monogr. 7:75-89] containing

- 5 10% FCS and 400 $\mu\text{g}/\text{ml}$ G418. The presence of "parental" chromosomes in the hybrid cell lines was verified by *in situ* hybridization with species-specific probes using biotin-labeled human and hamster genomic DNA, and a mouse long interspersed repetitive DNA [pMCPE1.51].

10 5. Microcell fusion

- Microcell-mediated transfer of artificial chromosomes from EC3/7C5 cells to recipient cells was done according to Saxon *et al.* [(1985) Mol. Cell. Biol. 1:140-146] with the modifications of Goodfellow *et al.* [(1989) Techniques for mammalian genome transfer. In Genome Analysis a Practical Approach. K.E. Davies, ed., IRL Press, Oxford, Washington DC. pp.1-17] and Yamada *et al.* [(1990) Oncogene 5:1141-1147]. Briefly, 5×10^6 EC3/7C5 cells in a T25 flask were treated first with 0.05 $\mu\text{g}/\text{ml}$ colcemid for 48 hr and then with 10 $\mu\text{g}/\text{ml}$ cytochalasin B for 30 min. The T25 flasks were centrifuged on edge and the pellet d 20 microcells were suspended in serum free DME medium. The microcells were filtered through first a 5 micron and then a 3 micron polycarbonat filter, treated with 50 $\mu\text{g}/\text{ml}$ of phytohemagglutin, and used for polyethylene glycol mediated fusion with recipient cells. Selection of cells containing the MMCneo was started 48 hours after fusion in 25 medium containing 400-800 $\mu\text{g}/\text{ml}$ G418.

Microcells were also prepared from 1B3 and GHB42 donor cells as follows in order to be fused with E2D6K cells [a CHO K-20 cell line carrying the puromycin N-acetyltransferase gene, *i.e.*, the puromycin resistance gene, under the control of the SV40 early promoter]. The

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donor cells were seeded to achieve 60-75% confluence within 24-36 hours. After that time, the cells were arrested in mitosis by exposure to colchicine (10 µg/ml) for 12 or 24 hours to induce micronucleation. To promote micronucleation of GHB42 cells, the cells were exposed to
5 hypotonic treatment (10 min at 37°C). After colchicine treatment, or after colchicine and hypotonic treatment, the cells were grown in colchicine-free medium.

The donor cells were trypsinized and centrifuged and the pellets were suspended in a 1:1 Percoll medium and incubated for 30-40 min at
10 37°C. After the incubation, 1-3 × 10⁷ cells (60-70% micronucleation index) were loaded onto each Percoll gradient (each fusion was distributed on 1-2 gradients). The gradients were centrifuged at 19,000 rpm for 80 min in a Sorvall SS-34 rotor at 34-37°C. After centrifugation, two visible bands of cells were removed, centrifuged at
15 2000 rpm, 10 min at 4°C, resuspended and filtered through 8 µm pore size nucleopore filters.

The microcells prepared from the 1B3 and GHB42 cells were fused with E2D6K. The E2D6K cells were generated by CaPO₄ transfection of CHO K-20 cells with pCHTV2. Plasmid pCHTV2 contains the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal,
20 the Saccharomyces cerevisiae URA3 gene, 2.4- and 3.2-kb fragments of a Chinese hamster chromosome 2-specific satellite DNA (HC-2 satellite; see Fatyol *et al.* (1994) Nuc. Acids Res. **22**:3728-3736), two copies of the diphtheria toxin-A chain gene (one linked to the herpes simplex virus
25 thymidine kinase (HSV-TK) gene promoter and SV40 polyadenylation signal and the other linked to the HSV-TK promoter without a polyadenylation signal), the ampicillin-resistance gene and the ColE1 origin of replication. Following transfection, puromycin-resistant colonies were isolated. The presence of the pCHTV2 plasmid in the E2D6K cell

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line was confirmed by nucleic acid amplification of DNA isolated from the cells.

- The purified microcells were centrifuged as described above and resuspended in 2 ml of phytohemagglutinin-P (PHA-P, 100 µg/ml). The 5 microcell suspension was then added to a 60-70% confluent recipient culture of E2D6K cells. The preparation was incubated at room temperature for 30-40 min to agglutinate the microcells. After the PHA-P was removed, the cells were incubated with 1 ml of 50% polyethylene-glycol (PEG) for one min. The PEG was removed and the culture was 10 washed three times with F-12 medium without serum. The cells were incubated in non-selective medium for 48-60 hours. After this time, the cell culture was trypsinized and plated in F-12 medium containing 400 µg/ml hygromycin B and 10 g/ml puromycin to select against the parental cell lines.
- 15 Hybrid clones were isolated from the cells that had been cultured in selective medium. These clones were then analyzed for expression of β-galactosidase by the X-gal staining method. Four of five hybrid clones analyzed that had been generated by fusion of GHB42 microcells with E2D6K cells yielded positive staining results indicating expression of β-galactosidase from the lacZ gene contained in the megachromosome contributed by the GHB42 cells. Similarly, a hybrid clone that had been generated by fusion of 1B3 microcells with E2D6K cells yielded positive staining results indicating expression of β-galactosidase from the lacZ gene contained in the megachromosome contributed by the 1B3 cells. *In* 20 *situ* hybridization analysis of the hybrid clones is also performed to analyze the mouse chromosome content of the mouse-hamster hybrid cells.
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B. Chromosome banding

Trypsin G-banding of chromosomes was performed using the method of Wang & Fedoroff [(1972) Nature 235:52-54], and the detection of constitutive heterochromatin with the BSG. C-banding 5 method was done according to Sumner [(1972) Exp. Cell Res. 75:304-306]. For the detection of chromosome replication by bromodeoxyuridine [BrdU] incorporation, the Fluorescein Plus Giemsa [FPG] staining method of Perry & Wolff [(1974) Nature 251:156-158] was used.

10 C. Immunolabelling of chromosomes and *in situ* hybridization

Indirect immunofluorescence labelling with human anti-centromere serum LU851 [Hadlaczky *et al.* (1986) Exp. Cell Res. 167:1-15], and indirect immunofluorescence and *in situ* hybridization on the same preparation were performed as described previously [see, Hadlaczky *et* 15 *al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:8106-8110, see, also U.S. application Serial No. 08/375,271]. Immunolabelling with fluorescein-conjugated anti-BrdU monoclonal antibody [Boehringer] was performed according to the procedure recommended by the manufacturer, except that for treatment of mouse A9 chromosomes, 2 M hydrochloric acid 20 was used at 37° C for 25 min, and for chromosomes of hybrid cells, 1 M hydrochloric acid was used at 37° C for 30 min.

D. Scanning electron microscopy

Preparation of mitotic chromosomes for scanning electron microscopy using osmium impregnation was performed as described 25 previously [Sumner (1991) Chromosoma 100:410-418]. The chromosomes were observed with a Hitachi S-800 field emission scanning electron microscope operated with an accelerating voltage of 25 kV.

E. DNA manipulations, plasmids and probes

1. General methods

- All general DNA manipulations were performed by standard procedures [see, e.g., Sambrook *et al.* (1989) *Molecular cloning: A 5 Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY]. The mouse major satellite probe was provided by Dr. J. B. Rattner [University of Calgary, Alberta, Canada]. Cloned mouse satellite DNA probes [see Wong *et al.* (1988) *Nucl. Acids Res.* **16**:11645-11661], including the mouse major satellite probe, were gifts from Dr. J. B. 10 Rattner, University of Calgary. Hamster chromosome painting was done with total hamster genomic DNA, and a cloned repetitive sequence specific to the centromeric region of chromosome 2 [Fátyol *et al.* (1994) *Nucl. Acids Res.* **22**:3728-3736] was also used. Mouse chromosome painting was done with a cloned long interspersed repetitive sequence 15 [pMCP1.51] specific for the mouse euchromatin.

For cotransfection and for *in situ* hybridization, the pCH110 β -galactosidase construct [Pharmacia or Invitrogen], and λ cI 875 Sam7 phage DNA [New England Biolabs] were used.

2. Construction of Plasmid pPuroTel

- 20 Plasmid pPuroTel, which carries a Puromycin-resistance gene and a cloned 2.5 kb human telomeric sequence [see SEQ ID No. 3], was constructed from the pBabe-puro retroviral vector [Morgenstern *et al.* (1990) *Nucl. Acids Res.* **18**:3587-3596; provided by Dr. L. Székely (Microbiology and Tumorbiology Center, Karolinska Institutet, 25 Stockholm); see, also Tonghua *et al.* (1995) *Chin. Med. J.* (Beijing, Engl. Ed.) **108**:653-659; Couto *et al.* (1994) *Infect. Immun.* **62**:2375-2378; Dunckley *et al.* (1992) *FEBS Lett.* **296**:128-34; French *et al.* (1995) *Anal. Biochem.* **228**:354-355; Liu *et al.* (1995) *Blood* **85**:1095-1103;

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International PCT application Nos. WO 9520044; WO 9500178, and WO 9419456].

F. Deposited cell lines

Cell lines KE1-2/4, EC3/7C5, TF1004G19C5, 19C5xHa4, G3D5

- 5 and H1D3 have been deposited in accord with the Budapest Treaty at the European Collection of Animal Cell Culture (ECACC) under Accession Nos. 96040924, 96040925, 96040926, 96040927, 96040928 and 96040929, respectively. The cell lines were deposited on April 9, 1996, at the European Collection of Animal Cell Cultures (ECACC) Vaccine 10 Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom. The deposits were made in the name of Gyula Hadlaczky of H. 6723, SZEGED, SZAMOS U.1.A. IX. 36. HUNGARY, who has authorized reference to the deposited cell lines in 15 this application and who has provided unreserved and irrevocable consent to the deposited cell lines being made available to the public in accordance with Rule 28(1)(d) of the European Patent Convention.

EXAMPLE 2

Preparation of EC3/7, EC3/7C5 and related cell lines

- 20 The EC3/7 cell line is an LMTK⁺ mouse cell line that contains the neo-centromere. The EC3/7C5 cell line is a single-cell subclone of EC3/7 that contains the neo-minichromosome.

A. EC3/7 Cell line

- As described in U.S. Patent No. 5,288,625 [see, also Praznovszky 25 et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88:11042-11046 and Hadlaczky et al. (1991) Proc. Natl. Acad. Sci. U.S.A. 88:8106-8110] *de novo* centromere formation occurs in a transformed mouse LMTK⁺ fibroblast cell line [EC3/7] after cointegration of λ constructs [λCM8 and λgtWESneo] carrying human and bacterial DNA.

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By cotransfection of a 14 kb human DNA fragment cloned in λ [λ CM8] and a dominant marker gene [λ gtWESneo], a selectable centromere linked to a dominant marker gene [neo-centromere] was formed in mouse LMTK cell line EC3/7 [Hadlaczky et al. (1991) Proc.

- 5 Natl. Acad. Sci. U.S.A. 88:8106-8110, see Figure 1]. Integration of the heterologous DNA [the λ DNA and marker gene-encoding DNA] occurred into the short arm of an acrocentric chromosome [chromosome 7 (see, Figure 1B)], where an amplification process resulted in the formation of the new centromere [neo-centromere (see Figure 1C)]. On the dicentric 10 chromosome (Figure 1C), the newly formed centromere region contains all the heterologous DNA (human, λ , and bacterial) introduced into the cell and an active centromere.

- Having two functionally active centromeres on the same chromosome causes regular breakages between the centromeres [see, 15 Figure 1E]. The distance between the two centromeres on the dicentric chromosome is estimated to be ~ 10-15 Mb, and the breakage that separates the minichromosome occurred between the two centromeres. Such specific chromosome breakages result in the appearance [in approximately 10% of the cells] of a chromosome fragment that carries 20 the neo-centromere [Figure 1F]. This chromosome fragment is principally composed of human, λ , plasmid, and neomycin-resistance gene DNA, but it also has some mouse chromosomal DNA. Cytological evidence suggests that during the stabilization of the MMCneo, there was an inverted duplication of the chromosome fragment bearing the 25 neo-centromere. The size of minichromosomes in cell lines containing the MMCneo is approximately 20-30 Mb; this finding indicates a two-fold increase in size.

From the EC3/7 cell line, which contains the dicentric chromosome [Figure 1E], two sublines [EC3/7C5 and EC3/7C6] were selected by

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repeated single-cell cloning. In these cell lines, the neo-centromere was found exclusively on a small chromosome [neo-minichromosome], while the formerly dicentric chromosome carried detectable amounts of the exogenously-derived DNA sequences but not an active neo-centromere
5 [Figure 1F and 1G].

The minichromosomes of cell lines EC3/7C5 and EC3/7C6 are similar. No differences are detected in their architectures at either the cytological or molecular level. The minichromosomes were indistinguishable by conventional restriction endonuclease mapping or by
10 long-range mapping using pulsed field electrophoresis and Southern hybridization. The cytoskeleton of cells of the EC3/7C6 line showed an increased sensitivity to colchicine, so the EC3/7C5 line was used for further detailed analysis.

B. Preparation of the EC3/7C5 and EC3/7C6 cell lines

15 The EC3/7C5 cells, which contain the neo-minichromosome, were produced by subcloning the EC3/7 cell line in high concentrations of G418 [40-fold the lethal dose] for 350 generations. Two single cell-derived stable cell lines [EC3/7C5 and EC3/7C6] were established. These cell lines carry the neo-centromere on minichromosomes and also
20 contain the remaining fragment of the dicentric chromosome. Indirect immunofluorescence with anti-centromere antibodies and subsequent in situ hybridization experiments demonstrated that the minichromosomes derived from the dicentric chromosome. In EC3/7C5 and EC3/7C6 cell lines (140 and 128 metaphases, respectively) no intact dicentric
25 chromosomes were found, and minichromosomes were detected in 97.2% and 98.1% of the cells, respectively. The minichromosomes have been maintained for over 150 cell generations. They do contain the remaining portion of the formerly dicentric chromosome.

Multiple copies of telomeric DNA sequences were detected in the marker centromeric region of the remaining portion of the formerly dicentric chromosome by *in situ* hybridization. This indicates that mouse telomeric sequences were coamplified with the foreign DNA sequences.

- 5 These stable minichromosome-carrying cell lines provide direct evidence that the extra centromere is functioning and is capable of maintaining the minichromosomes [see, U.S. Patent No. 5,288,625].

- The chromosome breakage in the EC3/7 cells, which separates the neo-centromere from the mouse chromosome, occurred in the G-band 10 positive "foreign" DNA region. This is supported by the observation of traces of λ and human DNA sequences at the broken end of the formerly dicentric chromosome. Comparing the G-band pattern of the 15 chromosome fragment carrying the neo-centromere with that of the stable neo-minichromosome, reveals that the neo-minichromosome is an inverted duplicate of the chromosome fragment that bears the neo-centromere. This is also evidenced by the observation that although the neo-minichromosome carries only one functional centromere, both ends of the minichromosome are heterochromatic, and mouse satellite DNA 20 sequences were found in these heterochromatic regions by *in situ* hybridization.

- These two cell lines, EC3/7C5 and EC3/7C6, thus carry a selectable mammalian minichromosome [MMCneo] with a centromere linked to a dominant marker gene [Hadlaczky *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. **88**:8106-8110]. MMCneo is intended to be used as a 25 vector for minichromosome-mediated gene transfer and has been used as model of a minichromosome-based vector system.

Long range mapping studies of the MMCneo indicated that human DNA and the neomycin-r sistance gene constructs integrated into the mouse chromosome separately, followed by the amplification of the

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chromosome region that contains the exogenous DNA. The MMCneo contains about 30-50 copies of the λ CM8 and λ gtWESneo DNA in the form of approximately 160 kb repeated blocks, which together cover at least a 3.5 Mb region. In addition to these, there are mouse telomeric sequences [Praznovszky *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:11042-11046] and any DNA of mouse origin necessary for the correct higher-ordered structural organization of chromatids.

Using a chromosome painting probe mCPE1.51 [mouse long interspersed repeated DNA], which recognizes exclusively euchromatic mouse DNA, detectable amounts of interspersed repeat sequences were found on the MMCneo by *in situ* hybridization. The neo-centromere is associated with a small but detectable amount of satellite DNA. The chromosome breakage that separates the neo-centromere from the mouse chromosome occurs in the "foreign" DNA region. This is demonstrated by the presence of λ and human DNA at the broken end of the formerly dicentric chromosome. At both ends of the MMCneo, however, there are traces of mouse major satellite DNA as evidenced by *in situ* hybridization. This observation suggests that the doubling in size of the chromosome fragment carrying the neo-centromere during the stabilization of the MMCneo is a result of an inverted duplication. Although mouse telomere sequences, which coamplified with the exogenous DNA sequences during the neo-centromere formation, may provide sufficient telomeres for the MMCneo, the duplication could have supplied the functional telomeres for the minichromosome.

The nucleotide sequence of portions of the neo-minichromosome was determined as follows. Total DNA was isolated from EC3/7C5 cells according to standard procedures. The DNA was subjected to nucleic acid amplification using the Expand Long Template PCR system [Boehringer Mannheim] according to the manufacturer's procedures. The

amplification procedure required only a single 33-mer oligonucleotide primer corresponding to sequence in a region of the phage λ right arm, which is contained in the neo-minichromosome. The sequence of this oligonucleotide is set forth as the first 33 nucleotides of SEQ ID No. 13.

- 5 Because the neo-minichromosome contains a series of inverted repeats of this sequence, the single oligonucleotide was used as a forward and reverse primer resulting in amplification of DNA positioned between sets of inverted repeats of the phage λ DNA. Three products were obtained from the single amplification reaction, which suggests that the sequence
- 10 of the DNA located between different sets of inverted repeats may differ. In a repeating nucleic acid unit within an artificial chromosome, minor differences may be present and may occur during culturing of cells containing the artificial chromosome. For example, base pair changes may occur as well as integration of mobile genetic elements and
- 15 deletions of repeated sequences.

- Each of the three products was subjected to DNA sequence analysis. The sequences of the three products are set forth in SEQ ID Nos. 13, 14, and 15, respectively. To be certain that the sequenced products were amplified from the neo-minichromosome, control amplifications were conducted using the same primers on DNA isolated from negative control cell lines (mouse Ltk⁻ cells) lacking minichromosomes and the formerly dicentric chromosome, and positive control cell lines [the mouse-hamster hybrid cell line GB43 generated by treating 19C5xHa4 cells (see Figure 4) with BrdU followed by growth in G418-containing selective medium and retreatment with BrdU] containing the neo-minichromosome only. Only the positive control cell line yielded the three amplification products; no amplification product was detected in the negative control reaction. The results obtained in the positive control amplification also demonstrate that the neo-minichromosome

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DNA, and not the fragment of the formerly dicentric mouse chromosome, was amplified.

The sequences of the three amplification products were compared to those contained in the Genbank/EMBL database. SEQ ID Nos. 13 and

5 14 showed high (~ 96%) homology to portions of DNA from intracisternal A-particles from mouse. SEQ ID No. 15 showed no significant homology with sequences available in the database. All three of these sequences may be used for generating gene targeting vectors as homologous DNAs to the neo-minichromosome.

10 C. Isolation and partial purification of minichromosomes

Mitotic chromosomes of EC3/7C5 cells were isolated as described by Hadlaczky *et al.* [(1981) Chromosoma 81:537-555], using a glycine-hexylene glycol buffer system [Hadlaczky *et al.* (1982)

15 Chromosoma 86:643-659]. Chromosome suspensions were centrifuged at 1,200 x g for 30 minutes. The supernatant containing minichromosomes was centrifuged at 5,000 x g for 30 minutes and the pellet was resuspended in the appropriate buffer. Partially purified minichromosomes were stored in 50% glycerol at -20° C.

D. Stability of the MMCneo maintenance and *neo* expression

20 EC3/7C5 cells grown in non-selective medium for 284 days and then transferred to selective medium containing 400 µg/ml G418 showed a 96% plating efficiency (colony formation) compared to control cells cultured permanently in the presence of G418. Cytogenetic analysis indicated that the MMCneo is stably maintained at one copy per cell
25 under selective and non-selective culture conditions. Only two metaphases with two MMCneo were found in 2,270 metaphases analyzed.

South rn hybridization analysis showed no detectable changes in DNA restriction patterns, and similar hybridization intensities were

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observed with a *neo* probe when DNA from cells grown under selective or non-selective culture conditions were compared.

Northern analysis of RNA transcripts from the *neo* gene isolated from cells grown under selective and non-selective conditions showed 5 only minor and not significant differences. Expression of the *neo* gene persisted in EC3/7C5 cells maintained in F-12 medium free of G418 for 290 days under non-selective culture conditions. The long-term expression of the *neo* gene(s) from the minichromosome may be influenced by the nuclear location of the MMCneo. *In situ* hybridization 10 experiments revealed a preferential peripheral location of the MMCneo in the interphase nucleus. In more than 60% of the 2,500 nuclei analyses, the minichromosome was observed at the perimeter of the nucleus near the nuclear envelope.

EXAMPLE 3

15 Minichromosome transfer and production of the λ -neo-chromosome

A. Minichromosome transfer

The neo-minichromosome [referred to as MMCneo, FIG. 2C] has been used for gene transfer by fusion of minichromosome-containing cells [EC3/7C5 or EC3/7C6] with different mammalian cells, including 20 hamster and human. Thirty-seven stable hybrid cell lines have been produced. All established hybrid cell lines proved to be true hybrids as evidenced by *in situ* hybridization using biotinylated human, and hamster genomic, or pMCPE1.51 mouse long interspersed repeated DNA probes for "chromosome painting". The MMCneo has also been successfully 25 transferred into mouse A9, L929 and pluripotent F9 teratocarcinoma cells by fusion of microcells derived from EC3/7C5 cells. Transfer was confirmed by PCR, Southern blotting and *in situ* hybridization with minichromosome-specific probes. The cytogenetic analysis confirmed

that, as expected for microcell fusion, a few cells [1-5%] received [or retained] the MMCneo.

These results demonstrate that the MMCneo is tolerated by a wide range of cells. The prokaryotic genes and the extra dosage for the

- 5 human and λ sequences carried on the minichromosome seem to be not disadvantageous for tissue culture cells.

The MMCneo is the smallest chromosome of the EC3/7C5 genome and is estimated to be approximately 20-30 Mb, which is significantly smaller than the majority of the host cell (mouse) chromosomes. By

- 10 virtue of the smaller size, minichromosomes can be partially purified from a suspension of isolated chromosomes by a simple differential centrifugation. In this way, minichromosome suspensions of 15-20% purity have been prepared. These enriched minichromosome preparations can be used to introduce, such as by microinjection or
15 lipofection, the minichromosome into selected target cells. Target cells include therapeutic cells that can be used in methods of gene therapy, and also embryonic cells for the preparation of transgenic (non-human) animals.

- The MMCneo is capable of autonomous replication, is stably
20 maintained in cells, and permits persistent expression of the *neo* gene(s), even after long-term culturing under non-selective conditions. It is a non-integrative vector that appears to occupy a territory near the nuclear envelope. Its peripheral localization in the nucleus may have an important role in maintaining the functional integrity and stability of the
25 MMCneo. Functional compartmentalization of the host nucleus may have an effect on the function of foreign sequences. In addition, MMCneo contains megabases of λ DNA sequences that should serve as a target site for homologous recombination and thus integration of desired gene(s) into the MMCneo. It can be transferred by cell and microcell

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fusion, microinjection, electroporation, lipid-mediated carrier systems or chromosome uptake. The neo-centromere of the MMCneo is capable of maintaining and supporting the normal segregation of a larger 150-200 Mb λ neo-chromosome. This result demonstrates that the MMCneo
5 chromosome should be useful for carrying large fragments of heterologous DNA.

B. Production of the λ neo-chromosome

In the hybrid cell line KE1-2/4 made by fusion of EC3/7 and Chinese hamster ovary cells [FIG 2], the separation of the neo-
10 centromere from the dicentric chromosome was associated with a further amplification process. This amplification resulted in the formation of a stable chromosome of average size [*i.e.*, the λ neo-chromosome; see, Praznovszky *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**:11042-11046]. The λ neo-chromosome carries a terminally located functional
15 centromere and is composed of seven large amplicons containing multiple copies of λ , human, bacterial, and mouse DNA sequences [see FIG 2]. The amplicons are separated by mouse major satellite DNA [Praznovszky *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**:11042-11046] which forms narrow bands of constitutive heterochromatin between the
20 amplicons.

EXAMPLE 4

Formation of the "sausage chromosome" [SC]

The findings set forth in the above EXAMPLES demonstrate that the centromeric region of the mouse chromosome 7 has the capacity for
25 large-scale amplification [other results indicate that this capacity is not unique to chromosome 7]. This conclusion is further supported by results from cotransfection experiments, in which a second dominant selectable marker gene and a non-selected marker gene were introduced into EC3/7C5 cells carrying the formerly dicentric chromosome 7 and the

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neo-minichromosome. The EC3/7C5 cell line was transformed with λ phage DNA, a hygromycin-resistance gene construct [pH132], and a β-galactosidase gene construct [pCH110]. Stable transformants were selected in the presence of high concentrations [400 µg/ml] Hygromycin
5 B, and analyzed by Southern hybridization. Established transformant cell lines showing multiple copies of integrated exogenous DNA were studied by *in situ* hybridization to localize the integration site(s), and by LacZ staining to detect β-galactosidase expression.

A. Materials and methods

10 1. Construction of pH132

The pH132 plasmid carries the hygromycin B resistance gene and the anti-HIV-1 *gag* ribozyme [see, SEQ ID NO. 6 for DNA sequence that corresponds to the sequence of the ribozyme] under control of the β-actin promoter. This plasmid was constructed from pHyg plasmid
15 [Sugden *et al.* (1985) *Mol. Cell. Biol.* 5:410-413; a gift from Dr. A. D. Riggs, Beckman Research Institute, Duarte; see, also, *e.g.*, U.S. Patent No. 4,997,764], and from pPC-RAG12 plasmid [see, Chang *et al.* (1990) *Clin Biotech* 2:23-31; provided by Dr. J. J. Rossi, Beckman Research Institute, Duarte; see, also U.S. Patent Nos. 5,272,262, 5,149,796 and
20 5,144,019, which describes the anti-HIV *gag* ribozyme and construction of a mammalian expression vector containing the ribozyme insert linked to the β-actin promoter and SV40 late gene transcriptional termination and polyA signals]. Construction of pPC-RAG12 involved insertion of the ribozyme insert flanked by BamHI linkers was into BamHI-digested pHβ-
25 Apr-1gpt [see, Gunning *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:4831-4835, see, also U.S. Patent No. 5,144,019].

Plasmid pH132 was constructed as follows. First, pPC-RAG12 [described by Chang *et al.* (1990) *Clin. Biotech.* 2:23-31] was digested with BamHI to excise a fragment containing an anti-HIV ribozyme gene

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[referred to as ribozyme D by Chang *et al.* [(1990) Clin. Biotech. 2:23-31]; see also U.S. Patent No. 5,144,019 to Rossi *et al.*... particularly Figure 4 of the patent] flanked by the human β -actin promoter at the 5' end of the gene and the SV40 late transcriptional termination and 5 polyadenylation signals at the 3' end of the gene. As described by Chang *et al.* [(1990) Clin. Biotech. 2:23-31], ribozyme D is targeted for cleavage of the translational initiation region of the HIV gag gene. This fragment of pPC-RAG12 was subcloned into pBluescript-KS(+) [Stratagene, La Jolla, CA] to produce plasmid 132. Plasmid 132 was 10 then digested with XbaI and EcoRI to yield a fragment containing the ribozyme D gene flanked by the β -actin promoter at the 5' end and the SV40 termination and polyadenylation signals at the 3' end of the gene. This fragment was ligated to the largest fragment generated by digestion of pHyg [Sugden *et al.* (1985) Mol. Cell. Biol. 5:410-413] with EcoRI and 15 SmaI to yield pH132. Thus, pH132 is an ~9.3 kb plasmid containing the following elements: the β -actin promoter linked to an anti-HIV ribozyme gene followed by the SV40 termination and polyadenylation signals, the thymidine kinase gene promoter linked to the hygromycin-resistance gene followed by the thymidine kinase gene polyadenylation signal, and the E. 20 coli ColE1 origin of replication and the ampicillin-resistance gene.

The plasmid pHyg [see, e.g., U.S. Patent Nos. 4,997,764, 4,686,186 and 5,162,215], which confers resistance to hygromycin B using transcriptional controls from the HSV-1 tk gene, was originally constructed from pKan2 [Yates *et al.* (1984) Proc. Natl. Acad. Sci. U.S.A. 81:3806-3810] and pLG89 [see, Gritz *et al.* (1983) Gene 25:179-188]. Briefly pKan2 was digested with SmaI and BglII to remove the sequences derived from transposon Tn5. The hygromycin-resistance hph gene was inserted into the digested pKan2 using blunt-end ligation at the SnaI site and "sticky-end" ligation [using 1 Weiss unit of T4 DNA

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ligase (BRL) in 20 microliter volume] at the BglIII site. The SmaI and BglIII sites of pKan2 were lost during ligation.

- The resulting plasmid pH132, produced from introduction of the anti-HIV ribozyme construct with promoter and polyA site into pHyg,
5 includes the anti-HIV ribozyme under control of the β -actin promoter as well as the hygromycin-resistance gene under control of the TK promoter.

2. Chromosome banding

- Trypsin G-banding of chromosomes was performed as described in
10 EXAMPLE 1.

3. Cell cultures

TF1004G19 and TF1004G-19C5 mouse cells and the 19C5xHa4 hybrid, described below, and its sublines were cultured in F-12 medium containing 400 μ g/ml Hygromycin B [Calbiochem].

15 B. Cotransfection of EC3/7C5 to produce TF1004G19

- Cotransfection of EC3/7C5 cells with plasmids [pH132, pCH110 available from Pharmacia, see, also Hall *et al.* (1983) *J. Mol. Appl. Gen.* 2:101-109] and with λ DNA [λ cI 875 Sam 7(New England Biolabs)] was conducted using the calcium phosphate DNA precipitation method [see, 20 *e.g.*, Chen *et al.* (1987) *Mol. Cell. Biol.* 7:2745-2752], using 2-5 μ g plasmid DNA and 20 μ g λ phage DNA per 5×10^6 recipient cells.

C. Cell lines containing the sausage chromosome

- Analysis of one of the transformants, designated TF1004G19, revealed that it has a high copy number of integrated pH132 and 25 pCH110 sequences, and a high level of β -galactosidase expression. G-banding and *in situ* hybridization with a human probe [CM8; see, *e.g.*, U.S. application Serial No. 08/375,271] revealed unexpectedly that integration had occurred in the formerly dicentric chromosome 7 of the EC3/7C5 cell line. Furthermore, this chromosome carried a newly formed

heterochromatic chromosome arm. The size of this heterochromatic arm varied between ~ 150 and ~ 800 Mb in individual metaphases.

By single cell cloning from the TF1004G19 cell line, a subclone TF1004G-19C5 [FIG 2D], which carries a stable chromosome 7 with a 5 ~ 100-150 Mb heterochromatic arm [the sausage chromosome] was obtained. This cell line has been deposited in the ECACC under Accession No. 96040926. This chromosome arm is composed of four to five satellite segments rich in satellite DNA, and evenly spaced integrat d heterologous "foreign" DNA sequences. At the end of the compact 10 heterochromatic arm of the sausage chromosome, a less condensed euchromatic terminal segment is regularly observed. This subclone was used for further analyses.

D. Demonstration that the sausage chromosome is derived from the formerly dicentric chromosome

15 *In situ* hybridization with λ phage and pH132 DNA on the TF1004G-19C5 cell line showed positive hybridization only on the minichromosome and on the heterochromatic arm of the "sausage" chromosome [Fig. 2D]. It appears that the "sausage" chromosome [herein also referred to as the SC] developed from the formerly dicentric 20 chromosome (FD) of the EC3/7C5 cell line.

To establish this, the integration sites of pCH110 and pH132 plasmids were determined. This was accomplished by *in situ* hybridization on these cells with biotin-labeled subfragments of the hygromycin-resistance gene and the β -galactosidase gene. Both 25 experiments resulted in narrow hybridizing bands on the heterochromatic arm of the sausage chromosome. The same hybridization pattern was detected on the sausage chromosome using a mixture of biotin-labeled λ probe and pH132 plasmid, proving the cointegration of λ phages, pH132 and pCH110 plasmids.

To examine this further, the cells were cultured in the presence of the DNA-binding dye Hoechst 33258. Culturing of mouse cells in the presence of this dye results in under-condensation of the pericentric heterochromatin of metaphase chromosomes, thereby permitting better

5 observation of the hybridization pattern. Using this technique, the heterochromatic arm of the sausage chromosome of TF1004G-19C5 cells showed regular under-condensation revealing the details of the structure of the "sausage" chromosome by *in situ* hybridization. Results of *in situ* hybridization on Hoechst-treated TF1004G-19C5 cells with biotin-labeled

10 subfragments of hygromycin-resistance and β -galactosidase genes shows that these genes are localized only in the heterochromatic arm of the sausage chromosome. In addition, an equal banding hybridization pattern was observed. This pattern of repeating units [amplicons] clearly indicates that the sausage chromosome was formed by an amplification

15 process and that the λ phage, pH132 and pCH110 plasmid DNA sequences border the amplicons.

In another series of experiments using fluorescence *in situ* hybridization [FISH] carried out with mouse major satellite DNA, the main component of the mouse pericentric heterochromatin, the results

20 confirmed that the amplicons of the sausage chromosome are primarily composed of satellite DNA.

E. The sausage chromosome has one centromere

To determine whether mouse centromeric sequences had participated in the amplification process forming the "sausage"

25 chromosome and whether or not the amplicons carry inactive centromeres, *in situ* hybridization was carried out with mouse minor satellite DNA. Mouse minor satellite DNA is localized specifically near the centromeres of all mouse chromosomes. Positive hybridization was detected in all mouse centromeres including the sausage chromosome,

which, however, only showed a positive signal at the beginning of the heterochromatic arm.

- Indirect immunofluorescence with a human anti-centromere antibody [LU 851] which recognizes only functional centromeres [see, 5 e.g., Hadlaczky et al. (1989) *Chromosoma* 97:282-288] proved that the sausage chromosome has only one active centromere. The centromere comes from the formerly dicentric part of the chromosome and co-localizes with the *in situ* hybridization signal of the mouse minor DNA probe.
- 10 F. The selected and non-selected heterologous DNA in the heterochromatin of the sausage chromosome is expressed
1. High levels of the heterologous genes are expressed
- The TF1004G-19C5 cell line thus carries multiple copies of hygromycin-resistance and β -galactosidase genes localized only in the 15 heterochromatic arm of the sausage chromosome. The TF1004G-19C5 cells can grow very well in the presence of 200 μ g/ml or even 400 μ g/ml hygromycin B. [The level of expression was determined by Northern hybridization with a subfragment of the hygromycin-resistance gene and single copy gene.]
- 20 The expression of the non-selected β -galactosidase gene in the TF1004G-19C5 transformant was detected with LacZ staining of the cells. By this method one hundred percent of the cells stained dark blue, showing that there is a high level of β -galactosidase expression in all of TF1004G-19C5 cells.
- 25 2. The heterologous genes that are expressed are in the heterochromatin of the sausage chromosome
- To demonstrate that the genes localized in the constitutive heterochromatin of the sausage chromosome provide the hygromycin resistance and the LacZ staining capability of TF1004G-19C5
- 30 transformants [i.e. β -gal expression], PEG-induced cell fusion between

TF1004G-19C5 mouse cells and Chinese hamster ovary cells was performed. The hybrids were selected and maintained in HAT medium containing G418 [400 µg/ml] and hygromycin [200 µg/ml]. Two hybrid clones designated 19C5xHa3 and 19C5xHa4, which have been 5 deposited in the ECACC under Accession No. 96040927, were selected. Both carry the sausage chromosome and the minichromosome.

Twenty-seven single cell derived colonies of the 19C5xHa4 hybrid were maintained and analyzed as individual subclones. *In situ* hybridization with hamster and mouse chromosome painting probes and 10 hamster chromosome 2-specific probes verified that the 19C5xHa4 clone contains the complete Chinese hamster genome and a partial mouse genome. All 19C5xHa4 subclones retained the hamster genome, but different subclones showed different numbers of mouse chromosomes indicating the preferential elimination of mouse chromosomes.

15 To promote further elimination of mouse chromosomes, hybrid cells were repeatedly treated with BrdU. The BrdU treatments, which destabilize the genome, result in significant loss of mouse chromosomes. The BrdU-treated 19C5xHa4 hybrid cells were divided to three groups. One group of the hybrid cells (GH) were maintained in the presence of 20 hygromycin (200 µg/ml) and G418 (400 µg/ml), and the other two groups of the cells were cultured under G418 (G) or hygromycin (H) selection conditions to promote the elimination of the sausage chromosome or minichromosome.

One month later, single cell derived subclones were established 25 from these three subcultures of the 19C5xHa4 hybrid line. The subclones were monitored by *in situ* hybridization with biotin-labeled λ phage and hamster chromosome painting probes. Four individual clones [G2B5, G3C5, G4D6, G2B4] selected in the presence of G418 that had lost the sausage chromosome but retained the minichromosome were

found. Under hygromycin selection only one subclone [G1C1] lost the minichromosome. In that clone the sausage chromosome [see Example 5] was present.

- Since hygromycin-resistance and β -galactosidase genes were
- 5 thought to be expressed from the sausage chromosome, the expression of these genes was analyzed in the four subclones that had lost the sausage chromosome. In the presence of 200 μ g/ml hygromycin, one hundred percent of the cells of four individual subclones died. In order to detect the β -galactosidase expression hybrid, subclones were analyzed
- 10 by LacZ staining. One hundred percent of the cells of the four subclones that lost the sausage chromosome also lost the LacZ staining capability. All of the other hybrid subclones that had not lost the sausage chromosome under the non-selective culture conditions showed positive LacZ staining.
- 15 These findings demonstrate that the expression of hygromycin-resistance and β -galactosidase genes is linked to the presence of the sausage chromosome. Results of *in situ* hybridizations show that the heterologous DNA is expressed from the constitutive heterochromatin of the sausage chromosome.
- 20 *In situ* hybridization studies of three other hybrid subclones [G2C6, G2D1, and G4D5] did not detect the presence of the sausage chromosome. By the LacZ staining method, some stained cells were detected in these hybrid lines, and when these subclones were transferred to hygromycin selection some colonies survived. Cytological
- 25 analysis and *in situ* hybridization of these hygromycin-resistant colonies revealed the presence of the sausage chromosome, suggesting that only the cells of G2C6, G2D1 and G4D5 hybrids that had not lost the sausage chromosome were able to preserve the hygromycin resistance and β -galactosidase expression. These results confirmed that the expression of

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these genes is linked to the presence of the sausage chromosome. The level of β -galactosidase expression was determined by the immunoblot technique using a monoclonal antibody.

- Hygromycin resistance and β -galactosidase expression of the cells which contained the sausage chromosome were provided by the genes localized in the mouse pericentric heterochromatin. This was demonstrated by performing Southern DNA hybridizations on the hybrid cells that lack the sausage chromosome using PCR-amplified subfragments of hygromycin-resistance and β -galactosidase genes as probes. None of the subclones showed hybridization with these probes; however, all of the analyzed clones contained the minichromosome. Other hybrid clones that contain the sausage chromosome showed intense hybridization with these DNA probes. These results lead to the conclusion that hygromycin resistance and β -galactosidase expression of the cells that contain the sausage chromosome were provided by the genes localized in the mouse pericentric heterochromatin.

EXAMPLE 5

The gigachromosome

- As described in Example 4, the sausage chromosome was transferred into Chinese hamster cells by cell fusion. Using Hygromycin B/HAT and G418 selection, two hybrid clones 19C5xHa3 and 19C5xHa4 were produced that carry the sausage chromosome. *In situ* hybridization, using hamster and mouse chromosome-painting probes and a hamster chromosome 2-specific probe, verified that clone 19C5xHa4 contains a complete Chinese hamster genome as well as partial mouse genomes. Twenty-seven separate colonies of 19C5xHa4 cells were maintained and analyzed as individual subclones. Twenty-six out of 27 subclones contained a morphologically unchanged sausage chromosome.

In one subclone of the 19C5xHa3 cell line, 19C5xHa47 [see Fig. 2E], the heterochromatic arm of the sausage chromosome became unstable and showed continuous intrachromosomal growth. In extreme cases, the amplified chromosome arm exceeded 1000 Mb in size

5 (gigachromosome).

EXAMPLE 6

The stable megachromosome

A. Generation of cell lines containing the megachromosome

All 19C5xHa4 subclones retained a complete hamster genome, but 10 different subclones showed different numbers of mouse chromosomes, indicating the preferential elimination of mouse chromosomes. As described in Example 4, to promote further elimination of mouse chromosomes, hybrid cells were treated with BrdU, cultured under G418 (G) or hygromycin (H) selection conditions followed by repeated 15 treatment with 10^{-4} M BrdU for 16 hours and single cell subclones were established. The BrdU treatments appeared to destabilize the genome, resulting in a change in the sausage chromosome as well. A gradual increase in a cell population in which a further amplification had occurred was observed. In addition to the ~100-150 Mb heterochromatic arm of 20 the sausage chromosome, an extra centromere and a ~150-250 Mb heterochromatic chromosome arm were formed, which differed from those of mouse chromosome 7. By the acquisition of another euchromatic terminal segment, a new submetacentric chromosome (megachromosome) was formed. Seventy-nine individual subclones were 25 established from these BrdU-treated cultures by single-cell cloning: 42 subclones carried the intact megachromosome, 5 subclones carried the sausage chromosome, and in 32 subclones fragments or translocated segments of the megachromosome were observed. Twenty-six subclones that carried the megachromosome were cultured under non-

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selective conditions over a two-month period. In 19 out of 26 subclones, the megachromosome was retained. Those subclones which lost the megachromosomes all became sensitive to Hygromycin B and had no β -galactosidase expression, indicating that both markers were
5 linked to the megachromosome.

Two sublines (G3D5 and H1D3), which were chosen for further experiments, showed no changes in the morphology of the megachromosome during more than 100 generations under selective conditions. The G3D5 cells had been obtained by growth of 19C5xHa4
10 cells in G418-containing medium followed by repeated BrdU treatment, whereas H1D3 cells had been obtained by culturing 19C5xHa4 cells in hygromycin-containing medium followed by repeated BrdU treatment.

B. Structure of the megachromosome

The following results demonstrate that, apart from the euchromatic
15 terminal segments, the integrated foreign DNA (and as in the exemplified embodiments, rDNA sequence), the whole megachromosome is constitutive heterochromatin, containing a tandem array of at least 40 [\sim 7.5 Mb] blocks of mouse major satellite DNA [see Figures 2 and 3]. Four satellite DNA blocks are organized into a giant palindrome
20 [amplicon] carrying integrated exogenous DNA sequences at each end. The long and short arms of the submetacentric megachromosome contains 6 and 4 amplicons, respectively. It is of course understood that the specific organization and size of each component can vary among species, and also the chromosome in which the amplification
25 event initiates.

1. The megachromosome is composed primarily of heterochromatin

Except for the terminal regions and the integrated foreign DNA, the megachromosome is composed primarily of heterochromatin. This was

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demonstrated by C-banding of the megachromosome, which resulted in positive staining characteristic of constitutive heterochromatin. Apart from the terminal regions and the integrated foreign DNA, the whole megachromosome appears to be heterochromatic. Mouse major satellite

5 DNA is the main component of the pericentric, constitutive heterochromatin of mouse chromosomes and represents ~ 10% of the total DNA [Waring *et al.* (1966) *Science* 154:791-794]. Using a mouse major satellite DNA probe for *in situ* hybridization, strong hybridization was observed throughout the megachromosome, except for its terminal

10 regions. The hybridization showed a segmented pattern: four large blocks appeared on the short arm and usually 4-7 blocks were seen on the long arm. By comparing these segments with the pericentric regions of normal mouse chromosomes that carry ~ 15 Mb of major satellite DNA, the size of the blocks of major satellite DNA on the

15 megachromosome was estimated to be ~ 30 Mb.

Using a mouse probe specific to euchromatin [pMCPE1.51; a mouse long interspersed repeated DNA probe], positive hybridization was detected only on the terminal segments of the megachromosome of the H1D3 hybrid subline. In the G3D5 hybrids, hybridization with a hamster-specific probe revealed that several megachromosomes contained terminal segments of hamster origin on the long arm. This observation indicated that the acquisition of the terminal segments on these chromosomes happened in the hybrid cells, and that the long arm of the megachromosome was the recently formed one arm. When a mouse minor satellite probe was used, specific to the centromeres of mouse chromosomes [Wong *et al.* (1988) *Nucl. Acids Res.* 16:11645-11661], a strong hybridization signal was detected only at the primary constriction of the megachromosome, which colocalized with the positive

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immunofluorescence signal produced with human anti-centromere serum [LU 851].

In situ hybridization experiments with pH132, pCH110, and λ DNA probes revealed that all heterologous DNA was located in the gaps

- 5 between the mouse major satellite DNA segments. Each segment of mouse major satellite DNA was bordered by a narrow band of integrated heterologous DNA, except at the second segment of the long arm where a double band of heterologous DNA existed, indicating that the major satellite DNA segment was missing or considerably reduced in size here.
- 10 This chromosome region served as a useful cytological marker in identifying the long arm of the megachromosome. At a frequency of 10^{-4} , "restoration" of these missing satellite DNA blocks was observed in one chromatid, when the formation of a whole segment on one chromatid occurred.
- 15 After Hoechst 33258 treatment (50 μ g/ml for 16 hours), the megachromosome showed undercondensation throughout its length except for the terminal segments. This made it possible to study the architecture of the megachromosome at higher resolution. *In situ* hybridization with the mouse major satellite probe on undercondensed
- 20 megachromosomes demonstrated that the ~30 Mb major satellite segments were composed of four blocks of ~7.5 Mb separated from each other by a narrow band of non-hybridizing sequences [Figure 3]. Similar segmentation can be observed in the large block of pericentric heterochromatin in metacentric mouse chromosomes from the LMTK⁺ and
- 25 A9 cell lines.

2. The megachromosome is composed of segments containing two tandem ~7.5 Mb blocks followed by two inverted blocks

Because of the asymmetry in thymidine content between the two
30 strands of the DNA of the mouse major satellite, when mouse cells are

grown in the presence of BrdU for a single S phase, the constitutive heterochromatin shows lateral asymmetry after FPG staining. Also, in the 19C5xHa4 hybrids, the thymidine-kinase [Tk] deficiency of the mouse fibroblast cells was complemented by the hamster Tk gene,
5 permitting BrdU incorporation experiments.

A striking structural regularity in the megachromosome was detected using the FPG technique. In both chromatids, alternating dark and light staining that produced a checkered appearance of the megachromosome was observed. A similar picture was obtained by
10 labelling with fluorescein-conjugated anti-BrdU antibody. Comparing these pictures to the segmented appearance of the megachromosome showed that one dark and one light FPG band corresponded to one ~ 30 Mb segment of the megachromosome. These results suggest that the two halves of the ~ 30 Mb segment have an inverted orientation. This
15 was verified by combining *in situ* hybridization and immunolabelling of the incorporated BrdU with fluorescein-conjugated anti-BrdU antibody on the same chromosome. Since the ~ 30 Mb segments [or amplicons] of the megachromosome are composed of four blocks of mouse major satellite DNA, it can be concluded that two tandem ~ 7.5 Mb blocks are
20 followed by two inverted blocks within one segment.

Large-scale mapping of megachromosome DNA by pulsed-field electrophoresis and Southern hybridization with "foreign" DNA probes revealed a simple pattern of restriction fragments. Using endonucleases with none, or only a single cleavage site in the integrated foreign DNA
25 sequences, followed by hybridization with a *hyg* probe, 1-4 predominant fragments were detected. Since the megachromosome contains 10-12 amplicons with an estimated 3-8 copies of *hyg* sequences per amplicon (30-90 copies per megachromosome), the small number of hybridizing fragments indicates the homogeneity of DNA in the amplified segments.

3. Scanning electron microscopy of the megachromosome confirmed the above findings

The homogeneous architecture of the heterochromatic arms of the megachromosome was confirmed by high resolution scanning electron microscopy. Extended arms of megachromosomes, and the pericentric heterochromatic region of mouse chromosomes, treated with Hoechst 33258, showed similar structure. The constitutive heterochromatic regions appeared more compact than the euchromatic segments. Apart from the terminal regions, both arms of the megachromosome were completely extended, and showed faint grooves, which should correspond to the border of the satellite DNA blocks in the non-amplified chromosomes and in the megachromosome. Without Hoechst treatment, the grooves seemed to correspond to the amplicon borders on the megachromosome arms. In addition, centromeres showed a more compact, finely fibrous appearance than the surrounding heterochromatin.

4. The megachromosome of 1B3 cells contains rRNA gene sequence

The sequence of the megachromosome in the region of the sites of integration of the heterologous DNA was investigated by isolation of these regions through using cloning methods and sequence analysis of the resulting clones. The results of this analysis revealed that the heterologous DNA was located near mouse ribosomal RNA gene (*i.e.*, rDNA) sequences contained in the megachromosome.

25 a. Cloning of regions of the megachromosomes in which heterologous DNA had integrated

Megachromosomes were isolated from 1B3 cells (which were generated by repeated BrdU treatment and single cell cloning of H1xHE41 cells (see Figure 4) and which contain a truncated megachromosome) using fluorescence-activated cell sorting methods as

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described herein (see Example 10). Following separation of the SATACs (megachromosomes) from the endogenous chromosomes, the isolated megachromosomes were stored in GH buffer (100 mM glycine, 1% hexylene glycol, pH 8.4-8.6 adjusted with saturated calcium

- 5 hydroxide solution; see Example 10) and centrifuged into an agarose bed in 0.5 M EDTA.

Large-scale mapping of the megachromosome around the area of the site of integration of the heterologous DNA revealed that it is enriched in sequence containing rare-cutting enzyme sites, such as the 10 recognition site for NotI. Additionally, mouse major satellite DNA (which makes up the majority of the megachromosome) does not contain NotI recognition sites. Therefore, to facilitate isolation of regions of the megachromosome associated with the site of integration of the heterologous DNA, the isolated megachromosomes were cleaved with 15 NotI, a rare cutting restriction endonuclease with an 8-bp GC recognition site. Fragments of the megachromosome were inserted into plasmid pWE15 (Stratagene, La Jolla, California) as follows. Half of a 100- μ l low melting point agarose block (mega-plug) containing the isolated SATACs was digested with NotI overnight at 37°C. Plasmid pWE15 was similarly 20 digested with NotI overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 ligase. Ligation was conducted at 16°C overnight. Bacterial DH5 α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total 25 of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and was analyzed by Southern blot hybridization for the presence of DNA that hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but yet containing the pWE15 plasmid, would be detected. Any

clones containing insert DNA would be expected to contain non-satellite, GC-rich megachromosome DNA sequences located at the site of integration of the heterologous DNA. All colonies were positive for hybridizing DNA.

- 5 Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. These clones were designated as follows: 28 (~ 9-kb insert), 30 (~ 9-kb insert), 60 (~ 4-kb insert), 113 (~ 9-kb insert), 157 (~ 9-kb insert) and 161 (~ 9-kb
 10 insert). Restriction enzyme analysis indicated that three of the clones (113, 157 and 161) contained the same insert.

b. *In situ* hybridization experiments using isolated segments of the megachromosome as probes

- Insert DNA from clones 30, 113, 157 and 161 was purified,
 15 labeled and used as probes in *in situ* hybridization studies of several cell lines. Counterstaining of the cells with propidium iodide facilitated identification of the cytological sites of the hybridization signals. The locations of the signals detected within the cells are summarized in the following table:

CELL TYPE	PROBE	LOCATION OF SIGNAL
Human Lymphocyte (male)	No. 161	4-5 pairs of acrocentric chromosomes at centromeric regions.
Mouse Spleen	No. 161	Acrocentric ends of 4 pairs of chromosomes.
EC3/7C5 Cells	No. 161	Minichromosome and the end of the formerly dicentric chromosome. Pericentric heterochromatin of one of the metacentric mouse chromosomes. Centromeric region of some of the other mouse chromosomes.
K20 Chines Hamster Cells	No. 30	Ends of at least 6 pairs of chromosomes. An interstitial signal on a short chromosome.

CELL TYPE	PROBE	LOCATION OF SIGNAL
HB31 Cells (mouse-hamster hybrid cells derived from H1D3 cells by repeated BrdU treatment and single cell cloning which carries the megachromosome)	No. 30	Acrocentric ends of at least 12 pairs of chromosomes. Centromeres of certain chromosomes and the megachromosome. Borders of the amplicons of the megachromosome.
Mouse Spleen Cells	No. 30	Similar to signal observed for probe no. 161. Centromeres of 5 pairs of chromosomes. Weak cross-hybridization to pericentric heterochromatin.
HB31 Cells	No. 113	Similar to signal observed for probe no. 30.
Mouse Spleen Cells	No. 113	Centromeric region of 5 pairs of chromosomes.
K20 Cells	No. 113	At least 6 pairs of chromosomes. Weak signal at some telomeres and several interspersed signals.
Human Lymphocyte Cells (male)	No. 157	Similar to signal observed for probe no. 161.

c. Southern blot hybridization using isolated segments of the megachromosome as probes

- DNA was isolated from mouse spleen tissue, mouse LMTK⁺ cells,
- 5 K20 Chinese hamster ovary cells, EJ30 human fibroblast cells and H1D3 cells. The isolated DNA and lambda phage DNA, was subjected to Southern blot hybridization using inserts isolated from megachromosome clone nos. 30, 113, 157 and 161 as probes. Plasmid pWE15 was used as a negative control probe. Each of the four megachromosome clone
- 10 inserts hybridized in a multi-copy manner (as demonstrated by the intensity of hybridization and the number of hybridizing bands) to all of the DNA samples, exc pt the lambda phage DNA. Plasmid pWE15 hybridized to lambda DNA only.

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d. Sequence analysis of megachromosome clone no. 161

Megachromosome clone no. 161 appeared to show the strongest hybridization in the *in situ* and Southern hybridization experiments and 5 was chosen for analysis of the insert sequence. The sequence analysis was approached by first subcloning the insert of cosmid clone no. 161 to obtain five subclones as follows.

To obtain the end fragments of the insert of clone no. 161, the clone was digested with NotI and BamHI and ligated with NotI/BamHI.
10 digested pBluescript KS (Stratagene, La Jolla, California). Two fragments of the insert of clone no. 161 were obtained: a 0.2-kb and a 0.7-kb insert fragment. To subclone the internal fragment of the insert of clone no. 161, the same digest was ligated with BamHI-digested pUC19. Three fragments of the insert of clone no. 161 were obtained: a 0.6-kb,
15 a 1.8-kb and a 4.8-kb insert fragment.

The ends of all the subcloned insert fragments were first sequenced manually. However, due to their extremely high GC content, autoradiographs were difficult to interpret and sequencing was repeated using an ABI sequencer and the dye-terminator cycle protocol. A
20 comparison of the sequence data to sequences in the GENBANK database revealed that the insert of clone no. 161 corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564, which is provided as SEQ ID NO. 16 herein. The sequence
25 data obtained for the insert of clone no. 161 is set forth in SEQ ID NOS. 18-24. Specifically, the individual subclones corresponded to the following positions in GENBANK accession no. X82564 (i.e., SEQ ID NO. 16) and in SEQ ID NOS. 18-24:

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Subclone	Start	End	Site	SEQ ID No.
	in X82564			
161k1	7579	7755	<u>NotI, BamHI</u>	18
161m5	7756	8494	<u>BamHI</u>	19
5 161m7	8495	10231	<u>BamHI</u>	20 (shows only sequence corresponding to nt. 8495-8950), 21 (shows only sequence corresponding to nt. 9851- 10231)
161m12	10232	15000	<u>BamHI</u>	22 (shows only sequence corresponding to nt. 10232-10600), 23 (shows only sequence corresponding to nt. 14267-15000),
161k2	15001	15676	<u>NotI, BamHI</u>	24

- The sequence set forth in SEQ ID NOs. 18-24 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA. The results of the sequence analysis of clone no. 161, which reveal that it corresponds to rDNA, correlate with the appearance of the *in situ* hybridization signal it generated in human lymphocytes and mouse spleen cells. The hybridization signal was clearly observed on acrocentric chromosomes in these cells, and such types of chromosomes are known to include rDNA adjacent to the pericentric satellite DNA on the short arm of the chromosome. Furthermore, rRNA genes are highly conserved in mammals as supported by the cross-species hybridization of clone no. 161 to human chromosomal DNA.

To isolate amplification-replication control regions such as those found in rDNA, it may be possible to subject DNA isolated from megachromosome-containing cells, such as H1D3 cells, to nucleic acid amplification using, e.g., the polymerase chain reaction (PCR) with the following primers:

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amplification control element forward primer (1-30)

5'-GAGGAATTCCCCATCCCTAATCCAGATTGGTG-3' (SEQ ID NO. 25)

amplification control element reverse primer (2142-2111)

5'-AAACTGCAGGCCGAGCCACCTCTCTGTGTTG-3' (SEQ ID NO. 26)

5 origin of replication region forward primer (2116-2141)

5'-AGGAATTACAGAACAGAGAGGTGGCTCGGCCTGC-3' (SEQ ID NO. 27)

origin of replication region reverse primer (5546-5521)

5'-AGCCTGCAGGAAGTCATACTGGGGAGGTGGCCC-3' (SEQ ID NO. 28)

C. Summary of the formation of the megachromosome

- 10 Figure 2 schematically sets forth events leading to the formation of a stable megachromosome beginning with the generation of a dicentric chromosome in a mouse LMTK⁺ cell line: (A) A single E-type amplification in the centromeric region of the mouse chromosome 7 following transfection of LMTK⁺ cells with λ CM8 and λ gtWESneo generates the
- 15 neo-centromere linked to the integrated foreign DNA, and forms a dicentric chromosome. Multiple E-type amplification forms the λ neo-chromosome, which was derived from chromosome 7 and stabilized in a mouse-hamster hybrid cell line; (B) Specific breakage between the centromeres of a dicentric chromosome 7 generates a chromosome
- 20 fragment with the neo-centromere, and a chromosome 7 with traces of foreign DNA at the end; (C) Inverted duplication of the fragment bearing the neo-centromere results in the formation of a stable neo-minichromosome; (D) Integration of exogenous DNA into the foreign DNA region of the formerly dicentric chromosome 7 initiates H-type
- 25 amplification, and the formation of a heterochromatic arm. By capturing a euchromatic terminal segment, this new chromosome arm is stabilized in the form of the "sausage" chromosome; (E) BrdU treatment and/or drug selection appears to induce further H-type amplification, which results in the formation of an unstable gigachromosome: (F) Repeated

BrdU treatments and/or drug selection induce further H-type amplification including a centromere duplication, which leads to the formation of another heterochromatic chromosome arm. It is split off from the chromosome 7 by chromosome breakage and acquires a terminal

5 segment to form the stable megachromosome.

D. Expression of β -galactosidase and hygromycin transferase genes in cell lines carrying the megachromosome or derivatives thereof

The level of heterologous gene (i.e., β -galactosidase and hygromycin transferase genes) expression in cell lines containing the

10 megachromosome or a derivative thereof was quantitatively measured. The relationship between the copy-number of the heterologous genes and the level of protein expressed therefrom was also determined.

1. Materials and methods

a. Cell lines

15 Heterologous gene expression levels of H1D3 cells, carrying a 250-400 Mb megachromosome as described above, and mM2C1 cells, carrying a 50-60 Mb micro-megachromosome, were quantitatively evaluated. mM2C1 cells were generated by repeated BrdU treatment and single cell cloning of the H1xHe41 cell line (mouse-hamster-human hybrid

20 cell line carrying the megachromosome and a single human chromosome with CD4 and neo' genes; see Figure 4). The cell lines were grown under standard conditions in F12 medium under selective (120 μ g/ml hygromycin) or non-selective conditions.

b. Preparation of cell extract for β -galactosidase assays

25 Monolayers of mM2C1 or H1D3 cell cultures were washed three times with phosphate-buffered saline (PBS). Cells were scraped by rubber policemen and suspended and washed again in PBS. Washed cells were resuspended into 0.25 M Tris-HCl, pH 7.8, and disrupted by

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three cycles of freezing in liquid nitrogen and thawing at 37°C. The extract was clarified by centrifugation at 12,000 rpm for 5 min. at 4°C.

c. *β-galacosidase assay*

The *β*-galactosidase assay mixture contained 1 mM MgCl₂,

- 5 45 mM *β*-mercaptoethanol, 0.8 mg/ml o-nitrophenyl-*β*-D-galactopyranoside and 66 mM sodium phosphate, pH 7.5. After incubating the reaction mixture with the cell extract at 37°C for increasing time, the reaction was terminated by the addition of three volumes of 1M Na₂CO₃, and the optical density was measured at 420 nm. Assay mixture incubated
10 without cell extract was used as a control. The linear range of the reaction was determined to be between 0.1-0.8 OD₄₂₀. One unit of *β*-galactosidase activity is defined as the amount of enzyme that will hydrolyse 3 nmoles of o-nitrophenyl-*β*-D-galactopyranoside in 1 minute at 37°C.

15 d. *Preparation of cell extract for hygromycin phosphotransferase assay*

- Cells were washed as described above and resuspended into 20 mM Hepes buffer, pH 7.3, 100 mM potassium acetate, 5 mM Mg acetate and 2 mM dithiothreitol). Cells were disrupted at 0°C by six 10 sec bursts in an MSE ultrasonic disintegrator using a microtip probe. Cells
20 were allowed to cool for 1 min after each ultrasonic burst. The extracts were clarified by centrifuging for 1 min at 2000 rpm in a microcentrifuge.

e. *Hygromycin phosphotransferase assay*

- Enzyme activity was measured by means of the phosphocellulose paper binding assay as described by Haas and Dowding [(1975). Meth.
25 Enzymol. 43:611-628]. The cell extract was supplemented with 0.1 M ammonium chloride and 1 mM adenosine-γ-³²P-triphosphate (specific activity: 300 Ci/mmol). The reaction was initiated by the addition of 0.1 mg/ml hygromycin and incubated for increasing time at 37°C. The reaction was terminated by heating the samples for 5 min at 75°C in a

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water bath, and after removing the precipitated proteins by centrifugation for 5 min in a microcentrifuge, an aliquot of the supernatant was spotted on a piece of Whatman P-81 phosphocellulose paper (2 cm^2). After 30 sec at room temperature the papers are placed into 500 ml of hot (75°C) 5 distilled water for 3 min. While the radioactive ATP remains in solution under these conditions, hygromycin phosphate binds strongly and quantitatively to phosphocellulose. The papers are rinsed 3 times in 500 ml of distilled water and the bound radioactivity was measured in toluene scintillation cocktail in a Beckman liquid scintillation counter. Reaction 10 mixture incubated without added hygromycin served as a control.

f. **Determination of the copy-number of the heterologous genes**

DNA was prepared from the H1D3 and mM2C1 cells using standard purification protocols involving SDS lysis of the cells followed 15 by Proteinase K treatment and phenol/chloroform extractions. The isolated DNA was digested with an appropriate restriction endonuclease, fractionated on agarose gels, blotted to nylon filters and hybridized with a radioactive probe derived either from the β -galactosidase or the hygromycin phosphotransferase genes. The level of hybridization was 20 quantified in a Molecular Dynamics PhosphorImage Analyzer. To control the total amount of DNA loaded from the different cells lines, the filters were reprobed with a single copy gene, and the hybridization of β -galactosidase and hygromycin phosphotransferase genes was normalized to the single copy gene hybridization.

25 g. **Determination of protein concentration**

The total protein content of the cell extracts was measured by the Bradford colorimetric assay using bovine serum albumin as standard.

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2. Characterization of the β -galactosidase and hygromycin phosphotransferase activity expressed in H1D3 and mM2C1 cells

- In order to establish quantitative conditions, the most important kinetic parameters of β -galactosidase and hygromycin phosphotransferase activity have been studied. The β -galactosidase activity measured with this colorimetric assay was linear between the 0.1-0.8 OD₄₂₀ range both for the mM2C1 and H1D3 cell lines. The β -galactosidase activity was also proportional in both cell lines with the amount of protein added to the reaction mixture within 5-100 μ g total protein concentration range. The hygromycin phosphotransferase activity of mM2C1 and H1D3 cell lines was also proportional with the reaction time or the total amount of added cell extract under the conditions described for the β -galactosidase.
- 15 a. Comparison of β -galactosidase activity of mM2C1 and H1D3 cell lines
- Cell extracts prepared from logarithmically growing mM2C1 and H1D3 cell lines were tested for β -galactosidase activity, and the specific activities were compared in 10 independent experiments. The β -galactosidase activity of H1D3 cell extracts was 440 ± 25 U/mg total protein. Under identical conditions the β -galactosidase activity of the mM2C1 cell extracts was 4.8 times lower: 92 ± 13 U/mg total protein.
- 20 β -galactosidase activities of highly subconfluent, subconfluent and nearly confluent cultures of H1D3 and mM2C1 cell lines were also compared. In these experiments different numbers of logarithmic H1D3 and mM2C1 cells were seeded in constant volume of culture medium and grown for 3 days under standard conditions. No significant difference was found in the β -galactosidase specific activities of cell cultures grown at different cell densities, and the ratio of H1D3/mM2C1 β -galactosidase
- 25 specific activities was also similar for all three cell densities. In

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confluent, stationary cell cultures of H1D3 or mM2C1 cells, however, the expression of β -galactosidase significantly decreased due likely to cessation of cell division as a result of contact inhibition.

5 **b. Comparison of hygromycin phosphotransferase activity of H1D3 and mM2C1 cell lines**

The bacterial hygromycin phosphotransferase is present in a membrane-bound form in H1D3 or mM2C1 cell lines. This follows from the observation that the hygromycin phosphotransferase activity can be completely removed by high speed centrifugation of these cell extracts, 10 and the enzyme activity can be recovered by resuspending the high speed pellet.

The ratio of the enzyme's specific activity in H1D3 and mM2C1 cell lines was similar to that of β -galactosidase activity, i.e., H1D3 cells have 4.1 times higher specific activity compared with mM2C1 cells.

15 **c. Hygromycin phosphotransferase activity in H1D3 and mM2C1 cells grown under non-selective conditions**

The level of expression of the hygromycin phosphotransferase gene was measured on the basis of quantitation of the specific enzyme activities in H1D3 and mM2C1 cell lines grown under non-selective 20 conditions for 30 generations. The absence of hygromycin in the medium did not influence the expression of the hygromycin phosphotransferase gene.

25 **3. Quantitation of the number of β -galactosidase and hygromycin phosphotransferase gene copies in H1D3 and mM2C1 cell lines**

As described above, the β -galactosidase and hygromycin phosphotransferase genes are located only within the megachromosome, or micro-megachromosome in H1D3 and mM2C1 cells. Quantitative analysis of genomic Southern blots of DNA isolated from H1D3 and 30 mM2C1 cell lines with the PhosphorImage Analyzer revealed that the

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copy number of β -galactosidase genes integrated into the megachromosome is approximately 10 times higher in H1D3 cells than in mM2C1 cells. The copy-number of hygromycin phosphotransferase genes is approximately 7 times higher in H1D3 cells than in mM2C1 cells.

5 4. **Summary and conclusions of results of quantitation of heterologous gene expression in cells containing megachromosomes or derivatives thereof**

Quantitative determination of β -galactosidase activity of higher eukaryotic cells (e.g., H1D3 cells) carrying the bacterial β -galactosidase gene in heterochromatic megachromosomes confirmed the observed high-level expression of the integrated bacterial gene detected by cytological staining methods. It has generally been established in reports of studies of the expression of foreign genes in transgenic animals that although transgene expression shows correct tissue and developmental specificity, the level of expression is typically low and shows extensive position-dependent variability (i.e., the level of transgene expression depends on the site of chromosomal integration). It is generally assumed that the low-level transgene expression may be due to the absence of special DNA sequences which can insulate the transgene from the inhibitory effect of the surrounding chromatin and promote the formation of active chromatin structure required for efficient gene expression. Several cis-activating DNA sequence elements have been identified which can abolish this position-dependent variability, and can ensure high-level expression of the transgene locus activating region (LAR) sequences in higher eukaryotes and specific chromatin structure (scs) elements in lower eukaryotes (see, e.g., Eissenberg and Elgin (1991) Trends in Genet. 7:335-340). If these cis-acting DNA sequences are absent, the level of transgene expression is low and copy-number independent.

Although the bacterial β -galactosidase reporter gene contained in the heterochromatic megachromosomes of H1D3 and mM2C1 cells is

- driven by a potent eukaryotic promoter-enhancer element, no specific cis-acting DNA sequence element was designed and incorporated into the bacterial DNA construct which could function as a boundary element. Thus, the high-level β -galactosidase expression measured in these cells is
- 5 of significance, particularly because the β -galactosidase gene in the megachromosome is located in a long, compact heterochromatic environment, which is known to be able to block gene expression. The megachromosome appears to contain DNA sequence element(s) in association with the bacterial DNA sequences that function to override
- 10 the inhibitory effect of heterochromatin on gene expression.
- The specificity of the heterologous gene expression in the megachromosome is further supported by the observation that the level of β -galactosidase expression is copy-number dependent. In the H1D3 cell line, which carries a full-size megachromosome, the specific activity
- 15 of β -galactosidase is about 5-fold higher than in mM2C1 cells, which carry only a smaller, truncated version of the megachromosome. A comparison of the number of β -galactosidase gene copies in H1D3 and mM2C1 cell lines by quantitative hybridization techniques confirmed that the expression of β -galactosidase is copy-number dependent. The
- 20 number of integrated β -galactosidase gene copies is approximately 10-fold higher in the H1D3 cells than in mM2C1 cells. Thus, the cell line containing the greater number of copies of the β -galactosidase gene also yields higher levels of β -galactosidase activity, which supports the copy-number dependency of expression. The copy number dependency of the
- 25 β -galactosidase and hygromycin phosphotransferase enzyme levels in cell lines carrying different derivatives of the megachromosome indicates that neither the chromatin organization surrounding the site of integration of the bacterial genes, nor the heterochromatic environment of the megachromosome suppresses the expression of the genes.

- The relative amount of β -galactosidase protein expressed in H1D3 cells can be estimated based on the V_{max} of this enzyme [500 for homogeneous, crystallized bacterial β -galactosidase (Naider *et al.* (1972) *Biochemistry* 11:3202-3210)] and the specific activity of H1D3 cell 5 protein. A V_{max} of 500 means that the homogeneous β -galactosidase protein hydrolyzes 500 μ moles of substrate per minute per mg of enzyme protein at 37°C. One mg of total H1D3 cell protein extract can hydrolyze 1.4 μ moles of substrate per minute at 37°C, which means that 0.28% of the protein present in the H1D3 cell extract is β -galactosidase.
- 10 The hygromycin phosphotransferase is present in a membrane-bound form in H1D3 and mM2C1 cells. The tendency of the enzyme to integrate into membranes in higher eukaryotic cells may be related to its periplasmic localization in prokaryotic cells. The bacterial hygromycin phosphotransferase has not been purified to homogeneity; thus, its V_{max} 15 has not been determined. Therefore, no estimate can be made on the total amount of hygromycin phosphotransferase protein expressed in these cell lines. The 4-fold higher specific activity of hygromycin phosphotransferase in H1D3 cells as compared to mM2C1 cells, however, indicates that its expression is also copy number dependent.
- 20 The constant and high level expression of the β -galactosidase gene in H1D3 and mM2C1 cells, particularly in the absence of any selective pressure for the expression of this gene, clearly indicates the stability of the expression of genes carried in the heterochromatic megachromosomes. This conclusion is further supported by the observation that the 25 level of hygromycin phosphotransferase expression did not change when H1D3 and mM2C1 cells were grown under non-selective conditions. The consistent high-level, stable, and copy-number dependent expression of bacterial marker genes clearly indicates that the megachromosome is an ideal vector system for expression of foreign genes.

EXAMPLE 7

Summary of some of the cell lines with SATACS and minichromosomes that have been constructed

5 1. EC3/7-Derived cell lines

The LMTK⁻-derived cell line, which is a mouse fibroblast cell line, was transfected with λ CM8 and λ gtWESneo DNA [see, EXAMPLE 2] to produce transformed cell lines. Among these cell lines was EC3/7, deposited at the European Collection of Animal Cell Culture (ECACC) 10 under Accession No. 90051001 [see, U.S. Patent No. 5,288,625; see, also Hadlaczky *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. **88**:8106-8110 and U.S. application Serial No. 08/375,271]. This cell line contains the dicentric chromosome with the neo-centromere. Recloning and selection produced cell lines such as EC3/7C5, which are cell lines with the stable 15 neo-minichromosome and the formerly dicentric chromosome [see, Fig. 2C].

2. KE1-2/4 Cells

Fusion of EC3/7 with CHO-K20 cells and selection with G418/HAT produced hybrid cell lines, among these was KE1-2/4, which has been 20 deposited with the ECACC under Accession No. 96040924. KE1-2/4 is a stable cell line that contains the λ neo-chromosome [see, Fig. 2D; see, also U.S. Patent No. 5,288,625], produced by E-type amplifications. KE1-2/4 has been transfected with vectors containing λ DNA, selectable 25 markers, such as the puromycin-resistance gene, and genes of interest, such as p53 and the anti-HIV ribozyme gene. These vectors target the gene of interest into the λ neo-chromosome by virtue of homologous recombination with the heterologous DNA in the chromosome.

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3. C5pMCT53 Cells

The EC3/7C5 cell line has been co-transfected with pH132, pCH110 and λ DNA [see, EXAMPLE 2] as well as other constructs.

Various clones and subclones have been selected. For example

- 5 transformation with a construct that includes p53 encoding DNA, produced cells designated C5pMCT53.

4. TF1004G24 Cells

As discussed above, cotransfection of EC3/7C5 cells with plasmids [pH132, pCH110 available from Pharmacia, see, also Hall *et al.*,

- 10 (1983) *J. Mol. Appl. Gen.* 2:101-109] and with λ DNA [λ cI 875 Sam 7 (New England Biolabs)] produced transformed cells. Among these is TF1004G24, which contains the DNA encoding the anti-HIV ribozyme in the neo-minichromosome. Recloning of TF1004G24 produced numerous cell lines. Among these is the NHHL24 cell line. This cell line also has
15 the anti-HIV ribozyme in the neo-minichromosome and expresses high levels of β -gal. It has been fused with CHO-K20 cells to produce various hybrids.

5. TF1004G19-Derived cells

Recloning and selection of the TF1004G transformants produced

- 20 the cell line TF1004G19, discussed above in EXAMPLE 4, which contains the unstable sausage chromosome and the neo-minichromosome. Single cell cloning produced the TF1004G-19C5 [see Figure 4] cell line, which has a stable sausage chromosome and the neo-minichromosome. TF1004G-19C5 has been fused with CHO cells and
25 the hybrids grown under selective conditions to produce the 19C5xHa4 and 19C5xHa3 cell lines [see, EXAMPLE 4] and others. Recloning of the 19C5xHa3 cell line yielded a cell line containing a gigachromosome, i.e., cell line 19C5xHa47, see Figure 2E. BrdU treatment of 19C5xHa4 cells and growth under selective conditions [neomycin (G) and/or hygromycin

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(H)] has produced hybrid cell lines such as the G3D5 and G4D6 cell lines and others. G3D5 has the neo-minichromosome and the megachromosome. G4D6 has only the neo-minichromosome.

- Recloning of 19C5xHa4 cells in H medium produced numerous clones. Among these is H1D3 [see Figure 4], which has the stable megachromosome. Repeated BrdU treatment and recloning of H1D3 cells has produced the HB31 cell line, which has been used for transformations with the pTEMPUD, pTEMPPU, pTEMPPU3, and pCEPUR-132 vectors [see, Examples 12 and 14, below].
- H1D3 has been fused with a CD4⁺ Hela cell line that carries DNA encoding CD4 and neomycin resistance on a plasmid [see, e.g., U.S. Patent Nos. 5,413,914, 5,409,810, 5,266,600, 5,223,263, 5,215,914 and 5,144,019, which describe these Hela cells]. Selection with GH has produced hybrids, including H1xHE41 [see Figure 4], which carries the megachromosome and also a single human chromosome that includes the CD4neo construct. Repeated BrdU treatment and single cell cloning has produced cell lines with the megachromosome [cell line 1B3, see Figure 4]. About 25% of the 1B3 cells have a truncated megachromosome (~ 90-120 Mb). Another of these subclones, designated 2C5, was cultured on hygromycin-containing medium and megachromosome-free cell lines were obtained and grown in G418-containing medium. Recloning of these cells yielded cell lines such as IB4 and others that have a dwarf megachromosome (~ 150-200 Mb), and cell lines, such as I1C3 and mM2C1, which have a micro-megachromosome (~ 50-90 Mb). The micro-megachromosome of cell

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line mM2C1 has no telomeres; however, if desired, synthetic telomeres, such as those described and generated herein, may be added to the mM2C1 cell micro-megachromosomes. Cell lines containing smaller truncated megachromosomes, such as the mM2C1 cell line containing

- 5 the micro-megachromosome, can be used to generate even smaller megachromosomes, e.g., ~10-30 Mb in size. This may be accomplished, for example, by breakage and fragmentation of the micro-megachromosome in these cells through exposing the cells to X-ray irradiation, BrdU or telomere-directed in vivo chromosome fragmentation.

10

EXAMPLE 8

Replication of the megachromosome

The homogeneous architecture of the megachromosomes provides a unique opportunity to perform a detailed analysis of the replication of the 15 constitutive heterochromatin.

A. Materials and methods

1. Culture of cell lines

H1D3 mouse-hamster hybrid cells carrying the megachromosome [see, EXAMPLE 4] were cultured in F-12 medium containing 10% fetal 20 calf serum [FCS] and 400 µg/ml Hygromycin B [Calbiochem]. G3D5 hybrid cells [see, Example 4] were maintained in F-12 medium containing 10% FCS, 400 µg/ml Hygromycin B (Calbiochem), and 400 µg/ml G418 [SIGMA]. Mouse A9 fibroblast cells were cultured in F-12 medium supplemented with 10% FCS.

25 2. BrdU labelling

In typical experiments, 20-24 parallel semi-confluent cell cultures were set up in 10 cm Petri dishes. Bromodeoxyuridine (BrdU) (Fluka) was dissolved in distilled water alkalized with a drop of NaOH, to make a 10⁻² M stock solution. Aliquots of 10-50 µl of this BrdU stock solution

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were added to each 10 ml culture, to give a final BrdU concentration of 10-50 μ M. The cells were cultured in the presence of BrdU for 30 min, and then washed with warm complete medium, and incubated without BrdU until required. At this point, 5 μ g/ml colchicine was added to a 5 sample culture every 1 or 2 h. After 1-2 h colchicine treatment, mitotic cells were collected by "shake-off" and regular chromosome preparations were made for immunolabelling.

3. Immunolabelling of chromosomes and *in situ* hybridization

Immunolabelling with fluorescein-conjugated anti-BrdU monoclonal 10 antibody (Boehringer) was done according to the manufacturer's recommendations, except that for mouse A9 chromosomes, 2 M hydrochloric acid was used at 37° C for 25 min, while for chromosomes of hybrid cells, 1 M hydrochloric acid was used at 37° C for 30 min. *In situ* hybridization with biotin-labelled probes, and indirect 15 immunofluorescence and *in situ* hybridization on the same preparation, were performed as described previously [Hadlaczky *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. **88**:8106-8110, see, also U.S. Patent No. 5,288,625].

4. Microscopy

20 All observations and microphotography were made by using a Vanox AHBS (Olympus) microscope. Fujicolor 400 Super G or Fujicolor 1600 Super HG high-speed colour negatives were used for photographs.

B. Results

The replication of the megachromosome was analyzed by BrdU 25 pulse labelling followed by immunolabelling. The basic parameters for DNA labelling *in vivo* were first established. Using a 30-min pulse of 50 μ M BrdU in parallel cultures, samples were taken and fixed at 5 min intervals from the beginning of the pulse, and every 15 min up to 1 h after the removal of BrdU. Incorporated BrdU was detected by

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immunolabelling with fluorescein-conjugated anti-BrdU monoclonal antibody. At the first time point (5 min) 38% of the nuclei were labelled, and a gradual increase in the number of labelled nuclei was observed during incubation in the presence of BrdU, culminating in 46% in the 30-
5 min sample, at the time of the removal of BrdU. At further time points (60, 75, and 90 min) no significant changes were observed, and the fraction of labelled nuclei remained constant [44.5-46%].

These results indicate that (i) the incorporation of the BrdU is a rapid process, (ii) the 30 min pulse-time is sufficient for reliable labelling
10 of S-phase nuclei, and (iii) the BrdU can be effectively removed from the cultures by washing.

The length of the cell cycle of the H1D3 and G3D5 cells was estimated by measuring the time between the appearance of the earliest BrdU signals on the extreme late replicating chromosome segments and
15 the appearance of the same pattern only on one of the chromatids of the chromosomes after one completed cell cycle. The length of G2 period was determined by the time of the first detectable BrdU signal on prophase chromosomes and by the labelled mitoses method [Qastler *et al.* (1959) Exp. Cell Res. **17**:420-438]. The length of the S-phase was
20 determined in three ways: (i) on the basis of the length of cell cycle and the fraction of nuclei labelled during the 30-120 min pulse; (ii) by measuring the time between the very end of the replication of the extreme late replicating chromosomes and the detection of the first signal on the chromosomes at the beginning of S phase; (iii) by the labelled
25 mitoses method. In repeated experiments, the duration of the cell cycle was found to be 22-26 h, the S phase 10-14 h, and the G2 phase 3.5-4.5 h.

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Analyses of the replication of the megachromosome were made in parallel cultures by collecting mitotic cells at two hour intervals following two hours of colchicine treatment. In a repeat experiment, the same analysis was performed using one hour sample intervals and one hour 5 colchicine treatment. Although the two procedures gave comparable results, the two hour sample intervals were viewed as more appropriate since approximately 30% of the cells were found to have a considerably shorter or longer cell cycle than the average. The characteristic replication patterns of the individual chromosomes, especially some of 10 the late replicating hamster chromosomes, served as useful internal markers for the different stages of S-phase. To minimize the error caused by the different lengths of cell cycles in the different experiments, samples were taken and analyzed throughout the whole cell cycle until 15 the appearance of the first signals on one chromatid at the beginning of the second S-phase.

The sequence of replication in the megachromosome is as follows. At the very beginning of the S-phase, the replication of the megachromosome starts at the ends of the chromosomes. The first initiation of replication in an interstitial position can usually be detected at 20 the centromeric region. Soon after, but still in the first quarter of the S-phase, when the terminal region of the short arm has almost completed its replication, discrete initiation signals appear along the chromosome arms. In the second quarter of the S-phase, as replication proceeds, the BrdU-labelled zones gradually widen, and the checkered pattern of the 25 megachromosome becomes clear [see, e.g., Fig. 2F]. At the same time, pericentric regions of mouse chromosomes also show intense incorporation of BrdU. The replication of the megachromosome peaks at the end of the second quarter and in the third quarter of the S-phase. At the end of the third quarter, and at the very beginning of the last quarter

of the S-phase, the megachromosome and the pericentric heterochromatin of the mouse chromosomes complete their replication. By the end of S-phase, only the very late replicating segments of mouse and hamster chromosomes are still incorporating BrdU.

- 5 The replication of the whole genome occurs in distinct phases. The signal of incorporated BrdU increased continuously until the end of the first half of the S-phase, but at the beginning of the third quarter of the S-phase chromosome segments other than the heterochromatic regions hardly incorporated BrdU. In the last quarter of the S-phase, the
10 BrdU signals increased again when the extreme late replicating segments showed very intense incorporation.

Similar analyses of the replication in mouse A9 cells were performed as controls. To increase the resolution of the immunolabelling pattern, pericentric regions of A9 chromosomes were decondensed by
15 treatment with Hoechst 33258. Because of the intense replication of the surrounding euchromatic sequences, precise localization of the initial BrdU signal in the heterochromatin was normally difficult, even on undercondensed mouse chromosomes. On those chromosomes where the initiation signal(s) were localized unambiguously, the replication of
20 the pericentric heterochromatin of A9 chromosomes was similar to that of the megachromosome. Chromosomes of A9 cells also exhibited replication patterns and sequences similar to those of the mouse chromosomes in the hybrid cells. These results indicate that the replicators of the megachromosome and mouse chromosomes retained
25 their original timing and specificity in the hybrid cells.

By comparing the pattern of the initiation sites obtained after BrdU incorporation with the location of the integration sites of the "foreign" DNA in a detailed analysis of the first quarter of the S-phase, an attempt was made to identify origins of replication (initiation sites) in relation to

the amplicon structure of the megachromosome. The double band of integrated DNA on the long arm of the megachromosome served as a cytological marker. The results showed a colocalization of the BrdU and *in situ* hybridization signals found at the cytological level, indicating that

- 5 the "foreign" DNA sequences are in close proximity to the origins of replication, presumably integrated into the non-satellite sequences between the replicator and the satellite sequences [see, Figure 3]. As described in Example 6.B.4, the rDNA sequences detected in the megachromosome are also localized at the amplicon borders at the site of
- 10 integration of the "foreign" DNA sequences, suggesting that the origins of replication responsible for initiation of replication of the megachromosome involve rDNA sequences. In the pericentric region of several other chromosomes, dot-like BrdU signals can also be observed that are comparable to the initiation signals on the megachromosome.
- 15 These signals may represent similar initiation sites in the heterochromatic regions of normal chromosomes.

At a frequency of 10^{-4} , "uncontrolled" amplification of the integrated DNA sequences was observed in the megachromosome. Consistent with the assumption (above) that "foreign" sequences are in

- 20 proximity of the replicators, this spatially restricted amplification is likely to be a consequence of uncontrolled repeated firings of the replication origin(s) without completing the replication of the whole segment.

C. Discussion

- It has generally been thought that the constitutive heterochromatin of the pericentric regions of chromosomes is late replicating [see, e.g., Miller (1976) *Chromosoma* 55:165-170]. On the contrary, these experiments evidence that the replication of the heterochromatic blocks starts at a discrete initiation site in the first half of the S-phase and continues through approximately three-quarters of S-phase. This

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- difference can be explained in the following ways: (i) in normal chromosomes, actively replicating euchromatic sequences that surround the satellite DNA obscure the initiation signals, and thus the precise localization of initiation sites is obscured; (ii) replication of the
- 5 heterochromatin can only be detected unambiguously in a period during the second half of the S-phase, when the bulk of the heterochromatin replicates and most other chromosomal regions have already completed their replication, or have not yet started it. Thus, low resolution cytological techniques, such as analysis of incorporation of radioactively
- 10 labelled precursors by autoradiography, only detect prominent replication signals in the heterochromatin in the second half of S-phase, when adjacent euchromatic segments are no longer replicating.

In the megachromosome, the primary initiation sites of replication colocalize with the sites where the "foreign" DNA sequences and rDNA sequences are integrated at the amplicon borders. Similar initiation signals were observed at the same time in the pericentric heterochromatin of some of the mouse chromosomes that do not have "foreign" DNA, indicating that the replication initiation sites at the borders of amplicons may reside in the non-satellite flanking sequences

15 of the satellite DNA blocks. The presence of a primary initiation site at each satellite DNA doublet implies that this large chromosome segment is a single huge unit of replication [megareplicon] delimited by the primary initiation site and the termination point at each end of the unit. Several lines of evidence indicate that, within this higher-order replication unit,

20 "secondary" origins and replicons contribute to the complete replication of the megareplicon:

1. The total replication time of the heterochromatic regions of the megachromosome was ~9-11 h. At the rate of movement of replication forks, 0.5-5 kb per minute, that is typical of eukaryotic

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chromosomes [Kornberg *et al.* (1992) *DNA Replication*. 2nd. ed., New York: W.H. Freeman and Co, p. 474], replication of a ~ 1.5 Mb replicon would require 50-500 h. Alternatively, if only a single replication origin was used, the average replication speed would have to be 25 kb per 5 minute to complete replication within 10 h. By comparing the intensity of the BrdU signals on the euchromatic and the heterochromatic chromosome segments, no evidence for a 5- to 50-fold difference in the replication speed was found.

2. Using short BrdU pulse labelling, a single origin of replication 10 would produce a replication band that moves along the replicon, reflecting the movement of the replication fork. In contrast, a widening of the replication zone that finally gave rise to the checkered pattern of the megachromosome was observed, and within the replication period, the most intensive BrdU incorporation occurred in the second half of the 15 S-phase. This suggests that once the megareplicator has been activated, it permits the activation and firing of "secondary" origins, and that the replication of the bulk of the satellite DNA takes place from these "secondary" origins during the second half of the S-phase. This is supported by the observation that in certain stages of the replication of 20 the megachromosome, the whole amplicon can apparently be labelled by a short BrdU pulse.

Megareplicators and secondary replication origins seem to be under strict temporal and spatial control. The first initiation within the megachromosomes usually occurred at the centromere, and shortly 25 afterward all the megareplicators become active. The last segment of the megachromosome to complete replication was usually the second segment of the long arm. Results of control experiments with mouse A9 chromosomes indicate that replication of the heterochromatin of mouse chromosomes corresponds to the replication of the megachromosome

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amplicons. Therefore, the pre-existing temporal control of replication in the heterochromatic blocks is preserved in the megachromosome.

Positive [Hassan *et al.* (1994) J. Cell. Sci. **107**:425-434] and negative [Haase *et al.* (1994) Mol. Cell. Biol. **14**:2516-2524] correlations

- 5 between transcriptional activity and initiation of replication have been proposed. In the megachromosome, transcription of the integrated genes seems to have no effect on the original timing of the replication origins. The concerted, precise timing of the megareplicator initiations in the different amplicons suggests the presence of specific, *cis*-acting
10 sequences, origins of replication.

Considering that pericentric heterochromatin of mouse chromosomes contains thousands of short, simple repeats spanning 7-15 Mb, and the centromere itself may also contain hundreds of kilobases, the existence of a higher-order unit of replication seems probable. The
15 observed uncontrolled intrachromosomal amplification restricted to a replication initiation region of the megachromosome is highly suggestive of a rolling-circle type amplification, and provides additional evidence for the presence of a replication origin in this region.

- The finding that a specific replication initiation site occurs at the
20 boundaries of amplicons suggests that replication might play a role in the amplification process. These results suggest that each amplicon of the megachromosome can be regarded as a huge megareplicon defined by a primary initiation site [megareplicator] containing "secondary" origins of replication. Fusion of replication bubbles from different origins of bi-
25 directional replication [DePamphilis (1993) Ann. Rev. Biochem. **62**:29-63] within the megareplicon could form a giant replication bubble, which would correspond to the whole megareplicon. In the light of this, the formation of megabase-size amplicons can be accommodated by a replication-directed amplification mechanism. In H and E-type

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amplifications, intrachromosomal multiplication of the amplicons was observed [see, above EXAMPLES], which is consistent with the unequal sister chromatid exchange model. Induced or spontaneous unscheduled replication of a megareplicon in the constitutive heterochromatin may

- 5 also form new amplicon(s) leading to the expansion of the amplification or to the heterochromatic polymorphism of "normal" chromosomes. The "restoration" of the missing segment on the long arm of the megachromosome may well be the result of the re-replication of one amplicon limited to one strand.

- 10 Taken together, without being bound by any theory, a replication-directed mechanism is a plausible explanation for the initiation of large-scale amplifications in the centromeric regions of mouse chromosomes, as well as for the *de novo* chromosome formations. If specific [amplifier, i.e., sequences controlling amplification] sequences play a role in 15 promoting the amplification process, sequences at the primary replication initiation site [megareplicator] of the megareplicon are possible candidates.

- The presence of rRNA gene sequence at the amplicon borders near the foreign DNA in the megachromosome suggests that this sequence 20 contributes to the primary replication initiation site and participates in large-scale amplification of the pericentric heterochromatin in *de novo* formation of SATACs. Ribosomal RNA genes have an intrinsic amplification mechanism that provides for multiple copies of tandem genes. Thus, for purposes herein, in the construction of SATACs in 25 cells, rDNA will serve as a region for targeted integration, and as components of SATACs constructed in vitro.

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EXAMPLE 9

Generation of chromosomes with amplified regions derived from mouse chromosome 1

To show that the events described in EXAMPLES 2-7 are not unique to mouse chromosome 7 and to show that the EC7/3 cell line is not required for formation of the artificial chromosomes, the experiments have been repeated using different initial cell lines and DNA fragments. Any cell or cell line should be amenable to use or can readily be determined that it is not.

10 A. Materials

The LP11 cell line was produced by the "scrape-loading" transfection method [Fechheimer *et al.* (1987) Proc. Natl. Acad. Sci. U.S.A. 84:8463-8467] using 25 µg plasmid DNA for 5×10^6 recipient cells. LP11 cells were maintained in F-12 medium containing 3-15 µg/ml

15 Puromycin [SIGMA].

B. Amplification in LP11 cells

The large-scale amplification described in the above Examples is not restricted to the transformed EC3/7 cell line or to the chromosome 7 of mouse. In an independent transformation experiment, LMTK⁺ cells were transfected using the calcium phosphate precipitation procedure with a selectable puromycin-resistance gene-containing construct designated pPuroTel [see Example 1.E.2. for a description of this plasmid], to establish cell line LP11. Cell line LP11 carries chromosome(s) with amplified chromosome segments of different lengths (~ 150-600 Mb). Cytological analysis of the LP11 cells indicated that the amplification occurred in the pericentric region of the long arm of a submetacentric chromosome formed by Robertsonian translocation. This chromosome arm was identified by G-banding as chromosome 1. C-banding and *in situ* hybridization with mouse major satellite DNA probe showed that an

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E-type amplification had occurred: the newly formed region was composed of an array of euchromatic chromosome segments containing different amounts of heterochromatin. The size and C-band pattern of the amplified segments were heterogeneous. In several cells, the number 5 of these amplified units exceeded 50; single-cell subclones of LP11 cell lines, however, carry stable marker chromosomes with 10-15 segments and constant C-band patterns.

Sublines of the thymidine kinase-deficient LP11 cells (e.g., LP11-15P 1C5/7 cell line) established by single-cell cloning of LP11 cells were 10 transfected with a thymidine kinase gene construct. Stable TK⁺ transfectants were established.

EXAMPLE 10

Isolation of SATACS and other chromosomes with atypical base content and/or size

15 I. Isolation of artificial chromosomes from endogenous chromosomes

Artificial chromosomes, such as SATACs, may be sorted from endogenous chromosomes using any suitable procedures, and typically involve isolating metaphase chromosomes, distinguishing the artificial 20 chromosomes from the endogenous chromosomes, and separating the artificial chromosomes from endogenous chromosomes. Such procedures will generally include the following basic steps: (1) culture of a sufficient number of cells (typically about 2×10^7 mitotic cells) to yield, preferably on the order of 1×10^6 artificial chromosomes, (2) arrest of 25 the cell cycle of the cells in a stage of mitosis, preferably metaphase, using a mitotic arrest agent such as colchicine, (3) treatment of the cells, particularly by swelling of the cells in hypotonic buffer, to increase susceptibility of the cells to disruption, (4) by application of physical force to disrupt the cells in the presence of isolation buffers for 30 stabilization of the released chromosomes, (5) dispersal of chromosomes

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- in the presence of isolation buffers for stabilization of free chromosomes, (6) separation of artificial from endogenous chromosomes and (7) storage (and shipping if desired) of the isolated artificial chromosomes in appropriate buffers. Modifications and variations of the general
- 5 procedure for isolation of artificial chromosomes, for example to accommodate different cell types with differing growth characteristics and requirements and to optimize the duration of mitotic block with arresting agents to obtain the desired balance of chromosome yield and level of debris, may be empirically determined.
- 10 Steps 1-5 relate to isolation of metaphase chromosomes. The separation of artificial from endogenous chromosomes (step 6) may be accomplished in a variety of ways. For example, the chromosomes may be stained with DNA-specific dyes such as Hoeschst 33258 and chromomycin A₃ and sorted into artificial and endogenous chromosomes
- 15 on the basis of dye content by employing fluorescence-activated cell sorting (FACS). To facilitate larger scale isolation of the artificial chromosomes, different separation techniques may be employed such as swinging bucket centrifugation (to effect separation based on chromosome size and density) [see, e.g., Mendelsohn *et al.* (1968) Mol. Biol. 32:101-108], zonal rotor centrifugation (to effect separation on the basis of chromosome size and density) [see, e.g., Burki *et al.* (1973) Prep. Biochem. 3:157-182; Stubblefield *et al.* (1978) Biochem. Biophys. Res. Commun. 83:1404-1414, velocity sedimentation (to effect separation on the basis of chromosome size and shape) [see e.g., Collard
- 20 *et al.* (1984) Cytometry 5:9-19]. Immuno-affinity purification may also be employed in larger scale artificial chromosome isolation procedures. In this process, large populations of artificial chromosome-containing cells (asynchronous or mitotically enriched) are harvested en masse and the mitotic chromosomes (which can be released from the cells using

standard procedures such as by incubation of the cells in hypotonic buffer and/or detergent treatment of the cells in conjunction with physical disruption of the treated cells) are enriched by binding to antibodies that are bound to solid state matrices (e.g. column resins or 5 magnetic beads). Antibodies suitable for use in this procedure bind to condensed centromeric proteins or condensed and DNA-bound histone proteins. For example, autoantibody LU851 (see Hadlaczky *et al.* (1989). Chromosoma 97:282-288), which recognizes mammalian centromeres may be used for large-scale isolation of chromosomes prior to 10 subsequent separation of artificial from endogenous chromosomes using methods such as FACS. The bound chromosomes would be washed and eventually eluted for sorting. Immunoaffinity purification may also be used directly to separate artificial chromosomes from endogenous chromosomes. For example, SATACs may be generated in or transferred 15 to (e.g., by microinjection or microcell fusion as described herein) a cell line that has chromosomes that contain relatively small amounts of heterochromatin, such as hamster cells (e.g., V79 cells or CHO-K1 cells). The SATACs, which are predominantly heterochromatin, are then separated from the endogenous chromosomes by utilizing anti-heterochromatin 20 binding protein (*Drosophila* HP-1) antibody conjugated to a solid matrix. Such matrix preferentially binds SATACs relative to hamster chromosomes. Unbound hamster chromosomes are washed away from the matrix and the SATACs are eluted by standard techniques.

A. Cell lines and cell culturing procedures

25 In one isolation procedure, 1B3 mouse-hamster-human hybrid cells [see, Figure 4] carrying the megachromosome or the truncated megachromosome were grown in F-12 medium supplemented with 10% fetal calf serum, 150 µg/ml hygromycin B and 400 µg/ml G418. GHB42 [a cell line recloned from G3D5 cells] mouse-hamster hybrid cells carrying

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the megachromosome and the minichromosome were also cultured in F-12 medium containing 10% fetal calf serum, 150 µg/ml hygromycin B and 400 µg/ml G418. The doubling time of both cell lines was about 24-40 hours, typically about 32 hours.

- 5 Typically, cell monolayers are passaged when they reach about 60-80% confluence and are split every 48-72 hours. Cells that reach greater than 80% confluence senesce in culture and are not preferred for chromosome harvesting. Cells may be plated in 100-200 100-mm dishes at about 50-70% confluency 12-30 hours before mitotic arrest (see,
10 below).

Other cell lines that may be used as hosts for artificial chromosomes and from which the artificial chromosomes may be isolated include, but are not limited to, PtK1 (NBL-3) marsupial kidney cells (ATCC accession no. CCL35), CHO-K1 Chinese hamster ovary cells (ATCC accession no. CCL61), V79-4 Chinese hamster lung cells (ATCC accession no. CCL93), Indian muntjac skin cells (ATCC accession no. CCL157), LMTK(-) thymidine kinase deficient murine L cells (ATCC accession no. CCL1.3), Sf9 fall armyworm (*Spodoptera frugiperda*) ovary cells (ATCC accession no. CRL 1711) and any generated heterokaryon (hybrid) cell
15 lines, such as, for example, the hamster-murine hybrid cells described herein, that may be used to construct MACs, particularly SATACs.
20

Cell lines may be selected, for example, to enhance efficiency of artificial chromosome production and isolation as may be desired in large-scale production processes. For instance, one consideration in selecting
25 host cells may be the artificial chromosome-to-total chromosome ratio of the cells. To facilitate separation of artificial chromosomes from endogenous chromosomes, a higher artificial chromosome-to-total chromosome ratio might be desirable. For example, for H1D3 cells (a murine/hamster heterokaryon; see Figure 4), this ratio is 1:50, i.e., one

artificial chromosome (the megachromosome) to 50 total chromosomes. In contrast, Indian muntjac skin cells (ATCC accession no. CCL157) contain a smaller total number of chromosomes (a diploid number of chromosomes of 7), as do kangaroo rat cells (a diploid number of 5 chromosomes of 12) which would provide for a higher artificial chromosome-to-total chromosome ratio upon introduction of, or generation of, artificial chromosomes in the cells.

Another consideration in selecting host cells for production and isolation of artificial chromosomes may be size of the endogenous 10 chromosomes as compared to that of the artificial chromosomes. Size differences of the chromosomes may be exploited to facilitate separation of artificial chromosomes from endogenous chromosomes. For example, because Indian muntjac skin cell chromosomes are considerably larger than minichromosomes and truncated megachromosomes, separation of 15 the artificial chromosome from the muntjac chromosomes may possibly be accomplished using univariate (one dye, either Hoechst 33258 or Chromomycin A3) FACS separation procedures.

Another consideration in selecting host cells for production and isolation of artificial chromosomes may be the doubling time of the cells. 20 For example, the amount of time required to generate a sufficient number of artificial chromosome-containing cells for use in procedures to isolate artificial chromosomes may be of significance for large-scale production. Thus, host cells with shorter doubling times may be desirable. For instance, the doubling time of V79 hamster lung cells is about 9-10 hours 25 in comparison to the approximately 32-hour doubling time of H1D3 cells.

Accordingly, several considerations may go into the selection of host cells for the production and isolation of artificial chromosomes. It may be that the host cell selected as the most desirable for de novo formation of artificial chromosomes is not optimized for large-scale

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production of the artificial chromosomes generated in the cell line. In such cases, it may be possible, once the artificial chromosome has been generated in the initial host cell line, to transfer it to a production cell line more well suited to efficient, high-level production and isolation of the

5 artificial chromosome. Such transfer may be accomplished through several methods, for example through microcell fusion, as described herein, or microinjection into the production cell line of artificial chromosomes purified from the generating cell line using procedures such as described herein. Production cell lines preferably contain two or more

10 copies of the artificial artificial chromosome per cell.

B. Chromosome isolation

In general, cells are typically cultured for two generations at exponential growth prior to mitotic arrest. To accumulate mitotic 1B3 and GHB42 cells in one particular isolation procedure, 5 µg/ml colchicine 15 was added for 12 hours to the cultures. The mitotic index obtained was 60-80%. The mitotic cells were harvested by selective detachment by gentle pipetting of the medium on the monolayer cells. It is also possible to utilize mechanical shake-off as a means of releasing the rounded-up (mitotic) cells from the plate. The cells were sedimented by

20 centrifugation at 200 x g for 10 minutes.

Cells (grown on plastic or in suspension) may be arrested in different stages of the cell cycle with chemical agents other than colchicine, such as hydroxyurea, vinblastine, colcemid or aphidicolin. Chemical agents that arrest the cells in stages other than mitosis, such 25 as hydroxyurea and aphidicolin, are used to synchronize the cycles of all cells in the population and then are removed from the cell medium to allow the cells to proceed, more or less simultaneously, to mitosis at which time they may be harvested to disperse the chromosomes. Mitotic cells could be enriched for a mechanical shake-off (adherent cells). The

cell cycles of cells within a population of MAC-containing cells may also be synchronized by nutrient, growth factor or hormone deprivation which leads to an accumulation of cells in the G₀ or G₁ stage; readdition of nutrients or growth factors then allows the quiescent cells to re-enter the 5 cell cycle in synchrony for about one generation. Cell lines that are known to respond to hormone deprivation in this manner, and which are suitable as hosts for artificial chromosomes, include the Nb2 rat lymphoma cell line which is absolutely dependent on prolactin for stimulation of proliferation (see Gout *et al.* (1980) Cancer Res. **40**:2433-10 2436). Culturing the cells in prolactin-deficient medium for 18-24 hours leads to arrest of proliferation, with cells accumulating early in the G₁ phase of the cell cycle. Upon addition of prolactin, all the cells progress through the cell cycle until M phase at which point greater than 90% of the cells would be in mitosis (addition of colchicine could increase the 15 amount of the mitotic cells to greater than 95%). The time between reestablishing proliferation by prolactin addition and harvesting mitotic cells for chromosome separation may be empirically determined.

Alternatively, adherent cells, such as V79 cells, may be grown in roller bottles and mitotic cells released from the plastic surface by 20 rotating the roller bottles at 200 rpm or greater (Shwarchuk *et al.* (1993) Int. J. Radiat. Biol. **64**:601-612). At any given time, approximately 1% of the cells in an exponentially growing asynchronous population is in M-phase. Even without the addition of colchicine, 2×10^7 mitotic cells have been harvested from four 1750-cm² roller bottles after a 5-min spin 25 at 200 rpm. Addition of colchicine for 2 hours may increase the yield to 6×10^8 mitotic cells.

Several procedures may be used to isolate metaphase chromosomes from these cells, including, but not limited to, one based on a polyamine buffer system [Cram *et al.* (1990) Methods in Cell

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- Biology 33:377-382], one on a modified hexylene glycol buffer system [Hadlaczky et al. (1982) Chromosoma 86:643-651], one on a magnesium sulfate buffer system [Van den Engh et al. (1988) Cytometry 9:266-270 and Van den Engh et al. (1984) Cytometry 5:108], one on an acetic acid fixation buffer system [Stoehr et al. (1982) Histochemistry 74:57-61], and one on a technique utilizing hypotonic KCl and propidium iodide [Cram et al. (1994) XVII meeting of the International Society for Analytical Cytology, October 16-21, Tutorial IV Chromosome Analysis and Sorting with Commerical Flow Cytometers; Cram et al. (1990) Methods in Cell Biology 33:376].

1. Polyamine procedure

- In the polyamine procedure that was used in isolating artificial chromosomes from either 1B3 or GHB42 cells, about 10^7 mitotic cells were incubated in 10 ml hypotonic buffer (75 mM KCl, 0.2 mM spermine, 0.5 mM spermidine) for 10 minutes at room temperature to swell the cells. The cells are swollen in hypotonic buffer to loosen the metaphase chromosomes but not to the point of cell lysis. The cells were then centrifuged at 100 x g for 8 minutes, typically at room temperature. The cell pellet was drained carefully and about 10^7 cells were resuspended in 1 ml polyamine buffer [15 mM Tris-HCl, 20 mM NaCl, 80 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM β -mercaptoethanol, 0.1% digitonin, 0.2 mM Spermine, 0.5 mM spermidine] for physical dispersal of the metaphase chromosomes. Chromosomes were then released by gently drawing the cell suspension up and expelling it through a 22 G needle attached to a 3 ml plastic syringe. The chromosome concentration was about $1-3 \times 10^8$ chromosomes/ml.

The polyamine buffer isolation protocol is well suited for obtaining high molecular weight chromosomal DNA [Sillar and Young (1981) J. Histochem. Cytochem. 29:74-78; VanDilla et al. (1986) Biotechnology

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4:537-552; Bartholdi *et al.* (1988) In "Molecular Genetics of Mammalian Cells" (M. Goetszman, ed.), Methods in Enzymology 151:252-267. Academic Press, Orlando]. The chromosome stabilizing buffer uses the polyamines spermine and spermidine to stabilize chromosome structure 5 [Blumenthal *et al.* (1979) J. Cell Biol. 81:255-259; Lalande *et al.* (1985) Cancer Genet. Cytogenet. 23:151-157] and heavy metals chelators to reduce nuclease activity.

The polyamine buffer protocol has wide applicability, however, as with other protocols, the following variables must be optimized for each 10 cell type: blocking time, cell concentration, type of hypotonic swelling buffer, swelling time, volume of hypotonic buffer, and vortexing time. Chromosomes prepared using this protocol are typically highly condensed.

There are several hypotonic buffers that may be used to swell the 15 cells, for example buffers such as the following: 75 mM KCl; 75 mM KCl, 0.2 mM spermine, 0.5 mM spermidine; Ohnuki's buffer of 16.2 mM sodium nitrate, 6.5 mM sodium acetate, 32.4 mM KCl [Ohnuki (1965) Nature 208:916-917 and Ohnuki (1968) Chromosoma 25:402-428]; and a variation of Ohnuki's buffer that additionally contains 0.2 mM spermine 20 and 0.5 mM spermidine. The amount and hypotonicity of added buffer vary depending on cell type and cell concentration. Amounts may range from 2.5 - 5.5 ml per 10^7 cells or more. Swelling times may vary from 10-90 minutes depending on cell type and which swelling buffer is used.

The composition of the polyamine isolation buffer may also be 25 varied. For example, one modified buffer contains 15 mM Tris-HCl, pH 7.2, 70 mM NaCl, 80 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM beta-mercaptoethanol, 0.25% Triton-X, 0.2 mM spermine and 0.5 mM spermidine.

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- Chromosomal dispersal may also be accomplished by a variety of physical means. For example, cell suspension may be gently drawn up and expelled in a 3-ml syringe fitted with a 22-gauge needle [Cram *et al.* (1990) Methods in Cell Biology 33:377-382], cell suspension may be
- 5 agitated on a bench-top vortex [Cram *et al.* (1990) Methods in Cell Biology 33:377-382], cell suspension may be disrupted with a homogenizer [Sillar and Young (1981) J. Histochem. Cytochem. 29:74-78; Carrano *et al.* (1979) Proc. Natl. Acad. Sci. U.S.A. 76:1382-1384] and cell suspension may be disrupted with a bench-top ultrasonic bath
- 10 [Stoehr *et al.* (1982) Histochemistry 74:57-61].

2. Hexylene glycol buffer system

- In the hexylene glycol buffer procedure that was used in isolating artificial chromosomes from either 1B3 or GHB42 cells, about 8×10^6 mitotic cells were resuspended in 10 ml glycine-hexylene glycol buffer
- 15 [100 mM glycine, 1% hexylene glycol, pH 8.4-8.6 adjusted with saturated Ca-hydroxide solution] and incubated for 10 minutes at 37°C, followed by centrifugation for 10 minutes to pellet the nuclei. The supernatant was centrifuged again at 200 x g for 20 minutes to pellet the chromosomes. Chromosomes were resuspended in isolation buffer
- 20 (1-3 $\times 10^8$ chromosomes/ml).

The hexylene glycol buffer composition may also be modified. For example, one modified buffer contains 25 mM Tris-HCl, pH 7.2, 750 mM hexylene glycol, 0.5 mM CaCl₂, 1.0 mM MgCl₂ [Carrano *et al.* (1979) Proc. Natl. Acad. Sci. U.S.A. 76:1382-1384].

25 3. Magnesium-sulfate buffer system

This buffer system may be used with any of the methods of cell swelling and chromosomal dispersal, such as described above in connection with the polyamine and hexylene glycol buffer systems. In this procedure, mitotic cells are resuspended in the following buffer: 4.8

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mM HEPES, pH 8.0, 9.8 mM MgSO₄, 48 mM KCl, 2.9 mM dithiothreitol [Van den Engh *et al.* (1985) *Cytometry* 6:92 and Van den Engh *et al.* (1984) *Cytometry* 5:108].

4. Acetic acid fixation buffer system

- 5 This buffer system may be used with any of the methods of cell swelling and chromosomal dispersal, such as described above in connection with the polyamine and hexylene glycol buffer systems. In this procedure, mitotic cells are resuspended in the following buffer: 25 mM Tris-HCl, pH 3.2, 750 mM (1,6)-hexandiol, 0.5 mM CaCl₂, 1.0% acetic acid [Stoehr *et al.* (1982) *Histochemistry* 74:57-61].
- 10

5. KCl-propidium iodide buffer system

- This buffer system may be used with any of the methods of cell swelling and chromosomal dispersal, such as described above in connection with the polyamine and hexylene glycol buffer systems. In 15 this procedure, mitotic cells are resuspended in the following buffer: 25 mM KCl, 50 µg/ml propidium iodide, 0.33% Triton X-100, 333 µg/ml RNase [Cram *et al.* (1990) *Methods in Cell Biology* 33:376].

- The fluorescent dye propidium iodide is used and also serves as a chromosome stabilizing agent. Swelling of the cells in the hypotonic 20 medium (which may also contain propidium iodide) may be monitored by placing a small drop of the suspension on a microscope slide and observing the cells by phase/fluorescent microscopy. The cells should exclude the propidium iodide while swelling, but some may lyse prematurely and show chromosome fluorescence. After the cells have 25 been centrifuged and resuspended in the KCl-propidium iodide buffer system, they will be lysed due to the presence of the detergent in the buffer. The chromosomes may then be dispersed and then incubated at 37 °C for up to 30 minutes to permit the RNase to act. The chromosome preparation is then analyzed by flow cytometry. The propidium iodide

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- fluorescence can be excited at the 488 nm wavelength of an argon laser and detected through an OG 570 optical filter by a single photomultiplier tube. The single pulse may be integrated and acquired in an univariate histogram. The flow cytometer may be aligned to a CV of 2% or less
- 5 using small (1.5 μm diameter) microspheres. The chromosome preparation is filtered through 60 μm nylon mesh before analysis.

C. Staining of chromosomes with DNA-specific dyes

- Subsequent to isolation, the chromosome preparation was stained with Hoechst 33258 at 6 $\mu\text{g}/\text{ml}$ and chromomycin A3 at 200 $\mu\text{g}/\text{ml}$.
- 10 Fifteen minutes prior to analysis, 25 mM Na-sulphite and 10 mM Na-citrate were added to the chromosome suspension.

D. Flow sorting of chromosomes

- Chromosomes obtained from 1B3 and GHB42 cells and maintained were suspended in a polyamine-based sheath buffer (0.5 mM EGTA, 2.0
- 15 mM EDTA, 80 mM KCl, 70 mM NaCl, 15 mM Tris-HCl, pH 7.2, 0.2 mM spermine and 0.5 mM spermidine) [Sillar and Young (1981) J. Histochem. Cytochem. 29:74-78]. The chromosomes were then passed through a dual-laser cell sorter [FACStar Plus or FAXStar Vantage Becton Dickinson Immunocytometry System; other dual-laser sorters may also be
- 20 used, such as those manufactured by Coulter Electronics (Elite ESP) and Cytomation (MoFlo)] in which two lasers were set to excite the dyes separately, allowing a bivariate analysis of the chromosome by size and base-pair composition. Because of the difference between the base composition of the SATACs and the other chromosomes and the
- 25 resulting difference in interaction with the dyes, as well as size differences, the SATACs were separated from the other chromosomes.

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E. Storage of the sorted artificial chromosomes

Sorted chromosomes may be pelleted by centrifugation and resuspended in a variety of buffers, and stored at 4°C. For example, the isolated artificial chromosomes may be stored in GH buffer (100 mM 5 glycine, 1% hexylene glycol pH 8.4–8.6 adjusted with saturated Ca-hydroxide solution) [see, e.g., Hadlaczky *et al.* (1982) *Chromosoma* 86:643-659] for one day and embedded by centrifugation into agarose. The sorted chromosomes were centrifuged into an agarose bed and the plugs are stored in 500 mM EDTA at 4° C. Additional storage buffers 10 include CMB-I/polyamine buffer (17.5 mM Tris-HCl, pH 7.4, 1.1 mM EDTA, 50 mM epsilon-amino caproic acid, 5 mM benzamide-HCl, 0.40 mM spermine, 1.0 mM spermidine, 0.25 mM EGTA, 40 mM KCl, 35 mM NaCl) and CMB-II/polyamine buffer (100 mM glycine, pH 7.5, 78 mM hexylene glycol, 0.1 mM EDTA, 50 mM epsilon-amino caproic acid, 5 15 mM benzamide-HCl, 0.40 mM spermine, 1.0 mM spermidine, 0.25 mM EGTA, 40 mM KCl, 35 mM NaCl).

When microinjection is the intended use, the sorted chromosomes are stored in 30% glycerol at -20° C. Sorted chromosomes may also be stored without glycerol for short periods of time (3-6 days) in storage 20 buffers at 4°C. Exemplary buffers for microinjection include CBM-I (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 mM epsilon-amino caproic acid, 5 mM benzamide-HCl, 0.30 mM spermine, 0.75 mM spermidine), CBM-II (100 mM glycine, pH 7.5, 78 mM hexylene glycol, 0.1 mM EDTA, 50 mM epsilon-amino caproic acid, 5 mM benzamide-HCl, 0.30 mM 25 spermine, 0.75 mM spermidine).

For long-term storage of sorted chromosomes, the above buffers are preferably supplemented with 50% glycerol and stored at -20°C.

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F. Quality control

1. Analysis of the purity

The purity of the sorted chromosomes was checked by fluorescence *in situ* hybridization (FISH) with a biotin-labeled mouse 5 satellite DNA probe [see, Hadlaczky *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:8106-8110]. Purity of the isolated chromosomes was about 97—99%.

2. Characteristics of the sorted chromosomes

Pulsed field gel electrophoresis and Southern hybridization were 10 carried out to determine the size distribution of the DNA content of the sorted artificial chromosomes.

G. Functioning of the purified artificial chromosomes

To check whether their activity is preserved, the purified artificial chromosomes may be microinjected (using methods such as those 15 described in Example 13) into primary cells, somatic cells and stem cells which are then analyzed for expression of the heterologous genes carried by the artificial chromosomes, e.g., such as analysis for growth on selective medium and assays of β -galactosidase activity.

II. Sorting of mammalian artificial chromosome-containing microcells

A. Micronucleation

Cells were grown to 80—90% confluency in 4 T150 flasks.

Colcemid was added to a final concentration of 0.06 μ g/ml, and then incubated with the cells at 37°C for 24 hours.

B. Enucleation

25 Ten μ g/ml cytochalasin B was added and the resulting microcells were centrifuged at 15,000 rpm for 70 minutes at 28—33° C.

C. Purification of microcells by filtration

The microcells were purified using Swinnex filter units and Nucleopore filters [5 μ m and 3 μ m].

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D. Staining and sorting microcells

As above, the cells were stained with Hoechst and chromomycin A3 dyes. The microcells were sorted by cell sorter to isolate the microcells that contain the mammalian artificial chromosomes.

5 E. Fusion

The microcells that contain the artificial chromosome are fused, for example, as described in Example 1.A.5., to selected primary cells, somatic cells, embryonic stem cells to generate transgenic (non-human) animals and for gene therapy purposes, and to other cells to deliver the
10 chromosomes to the cells.

EXAMPLE 11

Introduction of mammalian artificial chromosomes into insect cells

Insect cells are useful hosts for MACs, particularly for use in the production of gene products, for a number of reasons, including:

- 15 1. A mammalian artificial chromosome provides an extra-genomic specific integration site for introduction of genes encoding proteins of interest [reduced chance of mutation in production system].
2. The large size of an artificial chromosome permits megabase size DNA integration so that genes encoding an entire pathway leading to
20 a protein or nonprotein of therapeutic value, such as an alkaloid [digitalis, morphine, taxol] can be accommodated by the artificial chromosome.
3. Amplification of genes encoding useful proteins can be accomplished in the artificial mammalian chromosome to obtain higher protein yields in insect cells.
- 25 4. Insect cells support required post-translational modifications (glycosylation, phosphorylation) essential for protein biological function.
5. Insect cells do not support mammalian viruses — eliminates cross-contamination of product with human infectious agents.

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6. The ability to introduce chromosomes circumvents traditional recombinant baculovirus systems for production of nutritional, industrial or medicinal proteins in insect cell systems.

7. The low temperature optimum for insect cell growth (28° C)
5 permits reduced energy cost of production.

8. Serum free growth medium for insect cells will result in lower production costs.

9. Artificial chromosome-containing cells can be stored indefinitely at low temperature.

10 10. Insect larvae will serve as biological factories for the production of nutritional, medicinal or industrial proteins by microinjection of fertilized insect eggs.

A. Demonstration that insect cells recognize mammalian promoters
Gene constructs containing a mammalian promoter, such as the

15 CMV promoter, linked to a detectable marker gene [*Renilla* luciferase gene (see, e.g., U.S. Patent No. 5,292,658 for a description of DNA encoding the *Renilla* luciferase, and plasmid pTZrLuc-1, which can provide the starting material for construction of such vectors, see also SEQ ID No. 10] and also including the simian virus 40 (SV40) promoter

20 operably linked to the β -galactosidase gene were introduced into the cells of two species *Trichoplusia ni* [cabbage looper] and *Bombyx mori* [silk worm].

After transferring the constructs into the insect cell lines either by electroporation or by microinjection, expression of the marker genes was 25 detected in luciferase assays (see e.g., Example 12.C.3) and in β -galactosidase assays (such as lacZ staining assays) after a 24-h incubation. In each case a positive result was obtained in the samples containing the genes which was absent in samples in which the genes were omitted. In addition, a *B. mori* β -actin promoter-*Renilla* luciferase

gene fusion was introduced into the *T. ni* and *B. mori* cells which yielded light emission after transfection. Thus, certain mammalian promoters function to direct expression of these marker genes in insect cells. Therefore, MACs are candidates for expression of heterologous genes in 5 insect cells.

B. Construction of vectors for use in insect cells and fusion with mammalian cells

1. Transform LMTK cells with expression vector with:
 - a. *B. mori* β -actin promoter— Hyg' selectable marker 10 gene for insect cells, and
 - b. SV40 or CMV promoters controlling a puromycin' selectable marker gene for mammalian cells.
 2. Detect expression of the mammalian promoter in LMTK cells (puromycin' LMTK cells)
 - 15 3. Use puromycin' cells in fusion experiments with *Bombyx* and *Trichoplusia* cells, select Hyg' cells.

C. Insertion of the MACs into insect cells

These experiments are designed to detect expression of a detectable marker gene [such as the β -galactosidase gene expressed 20 under the control of a mammalian promoter, such as pSV40] located on a MAC that has been introduced into an insect cell. Data indicate that β -gal was expressed.

Insect cells are fused with mammalian cells containing mammalian artificial chromosomes, e.g., the minichromosome [EC3/7C5] or the mini 25 and the megachromosome [such as GHB42, which is a cell line recloned from G3D5] or a cell line that carries only the megachromosome [such as H1D3 or a reclone therefrom]. Fusion is carried out as follows:

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1. mammalian + insect cells (50/50%) in log phase growth are mixed;

2. calcium/PEG cell fusion: (10 min — 0.5 h);

3. heterokaryons (+ 72 h) are selected.

5 The following selection conditions to select for insect cells that contain a MAC can be used: [+ = positive selection; - = negative selection]:

1. growth at 28° C (+ insect cells, - mammalian cells);

2. Graces insect cell medium [SIGMA] (- mammalian cells);

10 3. no exogenous CO₂ (- mammalian cells); and/or

4. antibiotic selection (Hyg or G418) (+ transformed insect cells).

Immediately following the fusion protocol, many heterokaryons [fusion events] are observed between the mammalian and each species of insect cells [up to 90% heterokaryons]. After growth [2+ weeks] on 15 insect medium containing G418 and/or hygromycin at selection levels used for selection of transformed mammalian cells, individual colonies are detected growing on the fusion plates. By virtue of selection for the antibiotic resistance conferred by the MAC and selection for insect cells, these colonies should contain MACs.

20 The *B. mori* β-actin gene promoter has been shown to direct expression of the β-galactosidase gene in *B. mori* cells and mammalian cells (e.g., EC3/7C5 cells). The *B. mori* β-actin gene promoter is, thus, particularly useful for inclusion in MACs generated in mammalian cells that will subsequently be transferred into insect cells because the

25 presence of any marker gene linked to the promoter can be determined in the mammalian and resulting insect cell lines.

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EXAMPLE 12

Preparation of chromosome fragmentation vectors and other vectors for targeted integration of DNA into MACs

- Fragmentation of the megachromosome should ultimately result in
- 5 smaller stable chromosomes that contain about 15 Mb to 50 Mb that will be easily manipulated for use as vectors. Vectors to effect such fragmentation should also aid in determination and identification of the elements required for preparation of an in vitro-produced artificial chromosome.
- 10 Reduction in the size of the megachromosome can be achieved in a number of different ways including: stress treatment, such as by starvation, or cold or heat treatment; treatment with agents that destabilize the genome or nick DNA, such as BrdU, coumarin, EMS and others; treatment with ionizing radiation [see, e.g., Brown (1992) Curr.
- 15 Opin. Genes Dev. 2:479-486]; and telomere-directed in vivo chromosome fragmentation [see, e.g., Farr et al. (1995) EMBO J. 14:5444-5454].
- A. Preparation of vectors for fragmentation of the artificial chromosome and also for targeted integration of selected gene products
- 20 1. Construction of pTEMPUD
- Plasmid pTEMPUD [see Figure 5] is a mouse homologous recombination "killer" vector for in vivo chromosome fragmentation, and also for inducing large-scale amplification via site-specific integration. With reference to Figure 5, the ~3,625-bp Sall-PstI fragment was
- 25 derived from the pBabe-puro retroviral vector [see, Morgenstern et al. (1990) Nucleic Acids Res. 18:3587-3596]. This fragment contains DNA encoding ampicillin resistance, the pUC origin of replication, and the puromycin N-acetyl transferase gene under control of the SV40 early promoter. The URA3 gene portion comes from the pYAC5 cloning vector
- 30 [SIGMA]. URA3 was cut out of pYAC5 with Sall-XbaI digestion, cloned

into pNEB193 [New England Biolabs], which was then cut with EcoRI-Sall and ligated to the Sall site of pBabepuro to produce pPU.

A 1293-bp fragment [see SEQ ID No. 1] encoding the mouse major satellite, was isolated as an EcoRI fragment from a DNA library produced
5 from mouse LMTK fibroblast cells and inserted into the EcoRI site of pPU to produce pMPU.

The TK promoter-driven diphtheria toxin gene [DT-A] was derived from pMC1DT-A [see, Maxwell *et al.* (1986) *Cancer Res.* **46**:4660-4666] by BglII-XbaI digestion and cloned into the pMC1neo poly A expression
10 vector [STRATAGENE, La Jolla, CA] by replacing the neomycin-resistance gene coding sequence. The TK promoter, DT-A gene and poly A sequence were removed from this vector, cohesive ends were filled with Klenow and the resulting fragment blunt end-ligated and ligated into the SnaBI [TACGTA] of pMPU to produce pMPUD.

15 The Huetel 2.5-kb fragment [see SEQ ID No.3] was inserted at the PstI site [see the 6100 PstI - 3625 PstI fragment on pTEMPUD] of pMPUD to produce pTEMPUD. This fragment includes a human telomere. It includes a unique BglII site [see nucleotides 1042-1047 of SEQ ID No.3], which will be used as a site for introduction of a synthetic
20 telomere that includes multiple repeats [80] of TTAGGG with BamHI and BglII ends for insertion into the BglII site which will then remain unique, since the BamHI overhang is compatible with the BglII site. Ligation of a BamHI fragment to a BglII destroys the BglII site, so that only a single BglII site will remain. Selection for the unique BglII site insures that the
25 synthetic telomere will be inserted in the correct orientation. The unique BglII site is the site at which the vector is linearized.

To generate a synthetic telomere made up of multiple repeats of the sequence TTAGGG, attempts were made to clone or amplify ligation products of 30-mer oligonucleotides containing repeats of the sequence. Two 30-mer oligonucleotides, one containing four repeats of TTAGGG
5 bounded on each end of the complete run of repeats by half of a repeat and the other containing five repeats of the complement AATCCC, were annealed. The resulting double-stranded molecule with 3-bp protruding ends, each representing half of a repeat, was expected to ligate with itself to yield concatamers of $n \times 30$ bp. However, this approach was
10 unsuccessful, likely due to formation of quadruplex DNA from the G-rich strand. Similar difficulty has been encountered in attempts to generate long repeats of the pentameric human satellite II and III units. Thus, it appears that, in general, any oligomer sequence containing periodically spaced consecutive series of guanine nucleotides is likely to form
15 undesired quadruplex formation that hinders construction of long double-stranded DNAs containing the sequence.

Therefore, in another attempt to construct a synthetic telomere for insertion into the BgIII site of pTEMPUD, the starting material was based on the complementary C-rich repeat sequence (i.e., AATCCC) which
20 would not be susceptible to quadruplex structure formation. Two plasmids, designated pTEL280110 and pTel280111, were constructed as follows to serve as the starting materials.

First, a long oligonucleotide containing 9 repeats of the sequence AATCCC (i.e., the complement of telomere sequence TTAGGG) in
25 reverse order bounded on each end of the complete run of repeats by half of a repeat (therefore, in essence, containing 10 repeats), and recognition sites for PstI and PalI restriction enzymes was synthesized using standard methods. The oligonucleotide sequence is as follows:
5'-AAACTGCAGGTTAATTAAACCTAACCCCTAACCCCTAACCCCTAAC

CCTAACCCCTAACCCCTAACCCCTAACCCGGGAT-3' (SEQ ID NO. 29)

A partially complementary short oligonucleotide of sequence

3'-TTGGGCCCTAGGCTTAAGG-5' (SEQ ID NO. 30)

was also synthesized. The oligonucleotides were gel-purified, annealed,

- 5 repaired with Klenow polymerase and digested with EcoRI and PstI. The resulting EcoRI/PstI fragment was ligated with EcoRI/PstI-digested pUC19. The resulting plasmid was used to transform E. coli DH5 α competent cells and plasmid DNA (pTel102) from one of the transformants surviving selection on LB/ampicillin was digested with
- 10 PacI, rendered blunt-ended by Klenow and dNTPs and digested with HindIII. The resulting 2.7-kb fragment was gel-purified.

Simultaneously, the same plasmid was amplified by the polymerase chain reaction using extended and more distal 26-mer M13 sequencing primers. The amplification product was digested with SmaI and HindIII, the double-stranded 84-bp fragment containing the 60-bp telomeric repeat (plus 24 bp of linker sequence) was isolated on a 6% native polyacrylamide gel, and ligated with the double-digested pTel102 to yield a 120-bp telomeric sequence. This plasmid was used to transform DH5 α cells. Plasmid DNA from two of the resulting 20 recombinants that survived selection on ampicillin (100 μ g/ml) was sequenced on an ABI DNA sequencer using the dye-termination method. One of the plasmids, designated pTel29, contained a sequence of 20 repeats of the sequence TTAGGG (i.e., 19 successive repeats of TTAGGG bounded on each end of the complete run of repeats with half 25 of a repeat). The other plasmid, designated pTel28, had undergone a deletion of 2 bp (TA) at the junction where the two sequences, each containing, in essence, 10 repeats of the TTAGGG sequence, that had been ligated to yield the plasmid. This resulted in a GGGTGGG motif at the junction in pT 128. This mutation provides a useful tag in telomere-

directed chromosome fragmentation experiments. Therefore, the pTel29 insert was amplified by PCR using pUC/M13 sequencing primers based on sequence somewhat longer and farther from the polylinker than usual as follows:

- 5 5'-GCCAGGGTTTCCCAGTCACGACGT-3' (SEQ ID NO. 31)
or in some experiments
 5'-GCTGCAAGGCGATTAAGTTGGTAAC-3' (SEQ ID NO. 32)
as the m13 forward primer, and
 5'-TATGTTGTGGAATTGTGAGCGGAT-3' (SEQ ID NO. 33)
- 10 as the m13 reverse primer.
The amplification product was digested with SmaI and HindIII. The resulting 144-bp fragment was gel-purified on a 6% native polyacrylamide gel and ligated with pTel28 that had been digested with PacI, blunt-ended with Klenow and dNTP and then digested with HindIII
- 15 to remove linker. The ligation yielded a plasmid designated pTel2801 containing a telomeric sequence of 40 repeats of the sequence TTAGGG in which one of the repeats (i.e., the 30th repeat) lacked two nucleotides (TA), due to the deletion that had occurred in pTel28, to yield a repeat as follows: TGGG.
- 20 In the next extension step, pTel2801 was digested with SmaI and HindIII and the 264-bp insert fragment was gel-purified and ligated with pTel2801 which had been digested with PacI, blunt-ended and digested with HindIII. The resulting plasmid was transformed into DH5 α cells and plasmid DNA from 12 of the resulting transformants that survived
- 25 selection on ampicillin was examined by restriction enzyme analysis for the presence of a 0.5-kb EcoRI/PstI insert fragment. Eleven of the recombinants contained the expected 0.5-kb insert. The inserts of two of the recombinants were sequenced and found to be as expected. These plasmids were designated pTel280110 and pTel280111. These

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plasmids, which are identical, both contain 80 repeats of the sequence TTAGGG, in which two of the repeats (i.e., the 30th and 70th repeats) lacked two nucleotides (TA), due to the deletion that had occurred in pTel28, to yield a repeat as follows: TGGG. Thus, in each of the cloning 5 steps (except the first), the length of the synthetic telomere doubled; that is, it was increasing in size exponentially. Its length was 60×2^n bp, wherein n is the number of extension cloning steps undertaken. Therefore, in principle (assuming *E. coli*, or any other microbial host, e.g., yeast, tolerates long tandem repetitive DNA), it is possible to assemble 10 any desirable size of safe telomeric repeats.

In a further extension step, pTel280110 was digested with PacI, blunt-ended with Klenow polymerase in the presence of dNTP, then digested with HindIII. The resulting 0.5-kb fragment was gel purified. Plasmid pTel280111 was cleaved with SmaI and HindIII and the 3.2-kb 15 fragment was gel-purified and ligated to the 0.5-kb fragment from pTel280110. The resulting plasmid was used to transform DH5 α cells. Plasmid DNA was purified from transformants surviving ampicillin selection. Nine of the selected recombinants were examined by restriction enzyme analysis for the presence of a 1.0-kb EcoRI/PstI 20 fragment. Four of the recombinants (designated pTlk2, pTlk6, pTlk7 and pTlk8) were thus found to contain the desired 960 bp telomere DNA insert sequence that included 160 repeats of the sequence TTAGGG in which four of the repeats lacked two nucleotides (TA), due to the deletion that had occurred in pTel28, to yield a repeat as follows: TGGG. 25 Partial DNA sequence analysis of the EcoRI/PstI fragment of two of these plasmids (i.e., pTlk2 and pTlk6), in which approximately 300 bp from both ends of the fragment were elucidated, confirmed that the sequence was composed of successive repeats of the TTAGGG sequence.

In order to add Pmel and BgIII sites to the synthetic telomere sequence, pTlk2 was digested with PacI and PstI and the 3.7-kb fragment (i.e., 2.7-kb pUC19 and 1.0-kb repeat sequence) was gel-purified and ligated at the PstI cohesive end with the following

5 oligonucleotide 5'-GGGTTAACAGATCTCTGCA-3' (SEQ ID NO. 34). The ligation product was subsequently repaired with Klenow polymerase and dNTP, ligated to itself and transformed into E. coli strain DH5 α . A total of 14 recombinants surviving selection on ampicillin were obtained. Plasmid DNA from each recombinant was able to be cleaved with BgIII

10 indicating that this added unique restriction site had been retained by each recombinant. Four of the 14 recombinants contained the complete 1-kb synthetic telomere insert, whereas the insert of the remaining 10 recombinants had undergone deletions of various lengths. The four plasmids in which the 1-kb synthetic telomere sequence remained intact

15 were designated pTlkV2, pTlkV5, pTlkV8 an pTlkV12. Each of these plasmids could also be digested with Pmel; in addition the presence of both the BgIII nad Pmel sites was verified by sequence analysis. Any of these four plasmids can be digested with BamHI and BgIII to release a fragment containing the 1-kb synthetic telomere sequence which is then

20 ligated with BgIII-digested pTEMPUD.

2. Use of pTEMPUD for in vivo chromosome fragmentation

Linearization of pTEMPUD by BgIII results in a linear molecule with a human telomere at one end. Integration of this linear fragment into the chromosome, such as the megachromosome in hybrid cells or any mouse

25 chromosome which contains repeats of the mouse major satellite sequence results in integration of the selectable marker puromycin-resistance gene and cleavage of the plasmid by virtue of the telomeric end. The DT gene prevents that entire linear fragment from integrating by random events, since upon integration and expression it is toxic.

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Thus random integration will be toxic, so site-directed integration into the targeted DNA will be selected. Such integration will produce fragment d chromosomes.

- The fragmented truncated chromosome with the new telomere will
- 5 survive, and the other fragment without the centromere will be lost. Repeated in vivo fragmentations will ultimately result in selection of the smallest functioning artificial chromosome possible. Thus, this vector can be used to produce minichromosomes from mouse chromosomes, or to fragment the megachromosome. In principle, this vector can be used
- 10 to target any selected DNA sequence in any chromosome to achieve fragmentation.

3. Construction of pTERPUD

- A fragmentation/targeting vector analogous to pTEMPUD for in vivo chromosome fragmentation, and also for inducing large-scale
- 15 amplification via site-specific integration but which is based on mouse rDNA sequence instead of mouse major satellite DNA has been designated pTERPUD. In this vector, the mouse major satellite DNA sequence of pTEMPUD has been replaced with a 4770-bp BamHI fragment of megachromosome clone 161 which contains sequence
- 20 corresponding to nucleotides 10,232-15,000 in SEQ ID NO. 16.

4. pHASPUD and pTEMPhu3

- Vectors that specifically target human chromosomes can be constructed from pTEMPUD. These vectors can be used to fragment specific human chromosomes, depending upon the selected satellite
- 25 sequence, to produce human minichromosomes, and also to isolate human centromeres.

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a. pHASPU3

To render pTEMPUD suitable for fragmenting human chromosomes, the mouse major satellite sequence is replaced with human satellite sequences. Unlike mouse chromosomes, each human 5 chromosome has a unique satellite sequence. For example, the mouse major satellite has been replaced with a human hexameric α -satellite [or alphoid satellite] DNA sequence. This sequence is an 813-bp fragment [nucleotide 232-1044 of SEQ ID No. 2] from clone pS12, deposited in the EMBL database under Accession number X60716, isolated from a 10 human colon carcinoma cell line Colo320 [deposited under Accession No. ATCC CCL 220.1]. The 813-bp alphoid fragment can be obtained from the pS12 clone by nucleic acid amplification using synthetic primers, each of which contains an EcoRI site, as follows:

GGGAAATTCA TGGGATGTTT CAGTTGA forward primer [SEQ ID No. 4]

15 CGAAAGTCCCC CCTAGGAGAT CTTAAGGA reverse primer [SEQ ID No. 5].

Digestion of the amplified product with EcoRI results in a fragment with EcoRI ends that includes the human α -satellite sequence. This sequence is inserted into pTEMPUD in place of the EcoRI fragment that contains the mouse major satellite to yield pHASPU3.

20 Vector pHASPU3 was linearized with BglII and used to transform EJ30 (human fibroblast) cells by scrape loading. Twenty-seven puromycin-resistant transformant strains were obtained.

b. pTEMPhu3

In pTEMPhu3, the mouse major satellite sequence is replaced by 25 the 3kb human chromosome 3-specific α -satellite from D3Z1 [deposited under ATCC Accession No. 85434; see, also Yrokov (1989) Cytogenet. Cell Genet. 51:1114].

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5. Use of the pTEMPHU3 to induce amplification on human chromosome #3

- Each human chromosome contains unique chromosome-specific alphoid sequence. Thus, pTEMPHU3, which is targeted to the
- 5 chromosome 3-specific α -satellite, can be introduced into human cells under selective conditions, whereby large-scale amplification of the chromosome 3 centromeric region and production of a *de novo* chromosome ensues. Such induced large-scale amplification provides a means for inducing *de novo* chromosome formation and also for in vivo
- 10 cloning of defined human chromosome fragments up to megabase size.

For example, the break-point in human chromosome 3 is on the short arm near the centromere. This region is involved in renal cell carcinoma formation. By targeting pTEMPHu3 to this region, the induced large-scale amplification may contain this region, which can then be

15 cloned using the bacterial and yeast markers in the pTEMPHu3 vector.

- The pTEMPHu3 cloning vector allows not only selection for homologous recombinants, but also direct cloning of the integration site in YACS. This vector can also be used to target human chromosome 3, preferably with a deleted short arm, in a mouse-human mono-
- 20 chromosomal microcell hybrid line. Homologous recombinants can be screened by nucleic acid amplification (PCR), and amplification can be screened by DNA hybridization, Southern hybridization, and *in situ* hybridization. The amplified region can be cloned into a YAC. This vector and these methods also permit a functional analysis of cloned
- 25 chromosome regions by reintroducing the cloned amplified region into mammalian cells.

B. Preparation of libraries in YAC vectors for cloning of centromeres and identification of functional chromosomal units

- Another method that may be used to obtain smaller-sized functional mammalian artificial chromosome units and to clone
- 5 centromeric DNA involves screening of mammalian DNA YAC vector-based libraries and functional analysis of potential positive clones in a transgenic mouse model system. A mammalian DNA library is prepared in a YAC vector, such as YRT2 [see Schedl *et al.* (1993) *Nuc. Acids Res.* 21:4783-4787], which contains the murine tyrosinase gene. The library
- 10 is screened for hybridization to mammalian telomere and centromere sequence probes. Positive clones are isolated and microinjected into pronuclei of fertilized oocytes of NMRI/Han mice following standard techniques. The embryos are then transferred into NMRI/Han foster mothers. Expression of the tyrosinase gene in transgenic offspring
- 15 confers an identifiable phenotype (pigmentation). The clones that give rise to tyrosinase-expressing transgenic mice are thus confirmed as containing functional mammalian artificial chromosome units.

- Alternatively, fragments of SATACs may be introduced into the YAC vectors and then introduced into pronuclei of fertilized oocytes of
- 20 NMRI/Han mice following standard techniques as above. The clones that give rise to tyrosinase-expressing transgenic mice are thus confirmed as containing functional mammalian artificial chromosome units, particularly centromeres.

C. Incorporation of Heterologous Genes into Mammalian Artificial Chromosomes through The Use of Homology Targeting Vectors

- 25 As described above, the use of mammalian artificial chromosomes for expression of heterologous genes obviates certain negative effects that may result from random integration of heterologous plasmid DNA into the recipient cell genome. An essential feature of the mammalian
- 30 artificial chromosome that makes it a useful tool in avoiding the negative

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effects of random integration is its presence as an extra-genomic gene source in recipient cells. Accordingly, methods of specific, targeted incorporation of heterologous genes exclusively into the mammalian artificial chromosome, without extraneous random integration into the 5 genome of recipient cells, are desired for heterologous gene expression from a mammalian artificial chromosome.

One means of achieving site-specific integration of heterologous genes into artificial chromosomes is through the use of homology targeting vectors. The heterologous gene of interest is subcloned into a 10 targeting vector which contains nucleic acid sequences that are homologous to nucleotides present in the artificial chromosome. The vector is then introduced into cells containing the artificial chromosome for specific site-directed integration into the artificial chromosome through a recombination event at sites of homology between the vector 15 and the chromosome. The homology targeting vectors may also contain selectable markers for ease of identifying cells that have incorporated the vector into the artificial chromosome as well as lethal selection genes that are expressed only upon extraneous integration of the vector into the recipient cell genome. Two exemplary homology targeting vectors, 20 λ CF-7 and p λ CF-7-DTA, are described below.

1. Construction of Vector λ CF-7

Vector λ CF-7 contains the cystic fibrosis transmembrane conductance regulator [CFTR] gene as an exemplary therapeutic molecule-encoding nucleic acid that may be incorporated into mammalian 25 artificial chromosomes for use in gene therapy applications. This vector, which also contains the puromycin-resistance gene as a selectable marker, as well as the Saccharomyces cerevisiae ura3 gene [orotidine-5-phosphate decarboxylase], was constructed in a series of steps as follows.

a. Construction of pURA

Plasmid pURA was prepared by ligating a 2.6-kb Sall/Xhol fragment from the yeast artificial chromosome vector pYAC5 [Sigma; see also Burke *et al.* (1987) *Science* **236**:806-812 for a description of YAC vectors as well as GenBank Accession no. U01086 for the complete sequence of pYAC5] containing the S. cerevisiae ura3 gene with a 3.3-kb Sall/Smal fragment of pHyg [see, *e.g.*, U.S. Patent Nos. 4,997,764, 4,686,186 and 5,162,215,.. and the description above]. Prior to ligation the Xhol end was treated with Klenow polymerase for blunt end ligation to the Smal end of the 3.3 kb fragment of pHyyg. Thus, pURA contains the S. cerevisiae ura3 gene, and the E. coli ColE1 origin of replication and the ampicillin-resistance gene. The uraE gene is included to provide a means to recover the integrated construct from a mammalian cell as a YAC clone.

15 b. Construction of pUP2

Plasmid pURA was digested with Sall and ligated to a 1.5-kb Sall fragment of pCEPUR. Plasmid pCEPUR is produced by ligating the 1.1 kb SnaBI-Nhal fragment of pBabe-puro [Morgenstern *et al.* (1990) *Nucl. Acids Res.* **18**:3587-3596; provided by Dr. L. Székely (Microbiology and Tumorbiology Center, Karolinska Institutet, Stockholm); see, also Tonghua *et al.* (1995) *Chin. Med. J.* (Beijing, Engl. Ed.) **108**:653-659; Couto *et al.* (1994) *Infect. Immun.* **62**:2375-2378; Dunckley *et al.* (1992) *FEBS Lett.* **296**:128-34; French *et al.* (1995) *Anal. Biochem.* **228**:354-355; Liu *et al.* (1995) *Blood* **85**:1095-1103; International PCT application Nos. WO 9520044; WO 9500178, and WO 9419456] to the Nhel-Nrul fragment of pCEP4 [Invitrogen].

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The resulting plasmid, pUP2, contains all the elements of pURA plus the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal from pCEPUR.

c. Construction of pUP-CFTR

- 5 The intermediate plasmid pUP-CFTR was generated in order to combine the elements of pUP2 into a plasmid along with the CFTR gene. First, a 4.5-kb Sall fragment of pCMV-CFTR that contains the CFTR-encoding DNA [see, also, Riordan *et al.* (1989) *Science* 245:1066-1073, U.S. Patent No. 5,240,846, and Genbank Accession no. M28668
- 10 for the sequence of the CFTR gene] containing the CFTR gene only was ligated to XbaI-digested pCEP4 [Invitrogen and also described herein] in order to insert the CFTR gene in the multiple cloning site of the Epstein Barr virus-based (EBV) vector pCEP4 [Invitrogen, San Diego, CA; see also Yates *et al.* (1985) *Nature* 313:812-815; see, also U.S. Patent No.
- 15 5,468,615] between the CMV promoter and SV40 polyadenylation signal. The resulting plasmid was designated pCEP-CFTR. Plasmid pCEP-CFTR was then digested with Sall and the 5.8-kb fragment containing the CFTR gene flanked by the CMV promoter and SV40 polyadenylation signal was ligated to Sall-digested pUP2 to generate
- 20 pUP-CFTR. Thus, pUP-CFTR contains all elements of pUP2 plus the CFTR gene linked to the CMV promoter and SV40 polyadenylation signal.

d. Construction of λ CF-7

- Plasmid pUP-CFTR was then linearized by partial digestion with EcoRI and the 13 kb fragment containing the CFTR gene was ligated
- 25 with EcoRI-digested Charon 4A λ [see Blattner *et al.* (1977) *Science* 196:161; Williams and Blattner (1979) *J. Virol.* 29:555 and Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory Press, Volume 1, Section 2.18, for descriptions of Charon 4A λ]. The resulting vector, λ CF8, contains the Charon 4A λ

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- bacteriophage left arm, the CFTR gene linked to the CMV promoter and SV40 polyadenylation signal, the ura3 gene, the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal, the thymidine kinase promoter [TK], the ColE1 origin of replication, the 5 ampicillan resistance gene and the Charon 4A λ bacteriophage right arm. The λ CF8 construct was then digested with XbaI and the resulting 27.1 kb was ligated to the 0.4kb XbaI/EcoRI fragment of pJBP86 [described below], containing the SV40 polyA signal and the EcoRI-digested Charon 10 4A λ right arm. The resulting vector λ CF-7 contains the Charon 4A λ left arm, the CFTR encoding DNA linked to the CMV promoter and SV40 polyA signal, the ura3 gene, the puromycin resistance gene linked to the SV40 promoter and polyA signal and the Charon 4A λ right arm. The λ DNA fragments provide encode sequences homologous to nucleotides present in the exemplary artificial chromosomes.
- 15 The vector is then introduced into cells containing the artificial chromosomes exemplified herein. Accordingly, when the linear λ CF-7 vector is introduced into megachromosome-carrying fusion cell lines, such as described herein, it will be specifically integrated into the megachromosome through recombination between the homologous 20 bacteriophage λ sequences of the vector and the artificial chromosome.
2. Construction of Vector λ CF-7-DTA
- Vector λ CF-7-DTA also contains all the elements contained in λ CF-7, but additionally contains a lethal selection marker, the diphtheria toxin-A (DT-A) gene as well as the ampicillin-resistance gene and an origin of 25 replication. This vector was constructed in a series of steps as follows.

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a. Construction of pJBP86

Plasmid pJBP86 was used in the construction of λCF-7, above. A 1.5-kb Sall fragment of pCEPUR containing the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal was ligated 5 to HindIII-digested pJB8 [see, e.g., Ish-Horowitz *et al.* (1981) *Nucleic Acids Res.* **9**:2989-2998; available from ATCC as Accession No. 37074; commercially available from Amersham, Arlington Heights, IL]. Prior to ligation the Sall ends of the 1.5 kb fragment of pCEPUR and th4 HindIII linearized pJB8 ends were treated with Klenow polymerase. The 10 resulting vector pJBP86 contains the puromycin resistance gene linked to the SV40 promoter and polyA signal, the 1.8 kb COS region of Charon 4A1, the ColE1 origin of replication and the ampicillin resistance gene.

b. Construction of pMEP-DTA

A 1.1-kb Xhol/Sall fragment of pMC1-DT-A [see, e.g., Maxwell *et* 15 *al.* (1986) *Cancer Res.* **46**:4660-4666] containing the diphtheria toxin-A gene was ligated to Xhol-digested pMEP4 [Invitrogen, San Diego, CA] to generate pMEP-DTA. To produce pMC1-DT-A, the coding region of the DTA gene was isolated as a 800 bp PstIHindIII fragment from p2249-1 and inserted into pMC1neopolyA [pMC1 available from Stratagene] in 20 place of the neo gene and under the control of the TK promoter. The resulting construct pMC1DT-A was digested with HindIII, the ends filled by Klenow and Sall linkers were ligated to produce a 1061 bp TK-DTA gene cassette with an Xhol end [5'] and a Sall end containing the 270 bp TK promoter and the ~790 bp DT-A fragment. This fragment was 25 ligated into Xhol-digested pMEP4 .

Plasmid pMEP-DTA thus contains the DT-A gene linked to the TK promoter and SV40, ColE1 origin of replication and the ampicillin-resistance gene.

c. Construction of pJB83-DTA9

Plasmid pJB8 was digested with HindIII and ClI and ligated with an oligonucleotide [see SEQ ID NOs. 7 and 8 for the sense and antisense strands of the oligonucleotide, respectively] to generate pJB83.

- 5 The oligonucleotide that was ligated to ClI/HindIII-digested pJB8 contained the recognition sites of SwI, PaI and SrI restriction endonucleases. These sites will permit ready linearization of the λ CF-7-DTA construct.

- Next, a 1.4-kb Xhol/SaII fragment of pMEP-DTA, containing the
10 DT-A gene was ligated to SaII-digested pJB83 to generate pJB83-DTA9.

d. Construction of λ CF-7-DTA

- The 12-bp overhangs of λ CF-7 were removed by Mung bean nuclease and subsequent T4 polymerase treatments. The resulting 41.1-kb linear λ CF-7 vector was then ligated to pFB83-DTA9 which had been
15 digested with ClI and treated with T4 polymerase. The resulting vector, λ CF-7-DTA, contains all the elements of λ CF-7 as well as the DT-A gene linked to the TK promoter and the SV40 polyadenylation signal, the 1.8 kB Charon 4A λ COS region, the ampicillin-resistance gene [from pJB83-DTA9] and the Col E1 origin of replication [from pJB83-DT9A].

20 D. Targeting vectors using luciferase markers: Plasmid pMCT-RUC

- Plasmid pMCT-RUC [14 kbp] was constructed for site-specific targeting of the *Renilla* luciferase [see, e.g., U.S. Patent Nos. 5,292,658 and 5,418,155 for a description of DNA encoding *Renilla* luciferase, and plasmid pTZrLuc-1, which can provide the starting material for
25 construction of such vectors] gene to a mammalian artificial chromosome. The relevant features of this plasmid are the *Renilla* luciferase gene under transcriptional control of the human cytomegalovirus immediate-early gene enhancer/promoter; the hygromycin-resistance gene a, positive selectable marker, under the

- transcriptional control of the thymidine kinase promoter. In particular, this plasmid contains plasmid pAG60 [see, e.g., U.S. Patent Nos. 5,118,620, 5,021,344, 5,063,162 and 4,946,952; see, also Colbert-Garapin *et al.* (1981) *J. Mol. Biol.* 150:1-14], which includes DNA (i.e.,
- 5 the neomycin-resistance gene) homologous to the minichromosome, as well as the *Renilla* and hygromycin-resistance genes, the HSV-tk gene under control of the tk promoter as a negative selectable marker for homologous recombination, and a unique HpaI site for linearizing the plasmid.
- 10 This construct was introduced, via calcium phosphate transfection, into EC3/7C5 cells [see, Lorenz *et al.* (1996) *J. Biolum. Chemilum.* 11:31-37]. The EC3/7C5 cells were maintained as a monolayer [see, Gluzman (1981) *Cell* 23:175-183]. Cells at 50% confluence in 100 mm Petri dishes were used for calcium phosphate transfection [see, Harper *et*
- 15 *al.* (1981) *Chromosoma* 83:431-439] using 10 µg of linearized pMCT-RUC per plate. Colonies originating from single transfected cells were isolated and maintained in F-12 medium containing hygromycin (300 µg/mL) and 10% fetal bovine serum. Cells were grown in 100 mm Petri dishes prior to the *Renilla* luciferase assay.
- 20 The *Renilla* luciferase assay was performed [see, e.g., Matthews *et al.* (1977) *Biochemistry* 16:85-91]. Hygromycin-resistant cell lines obtained after transfection of EC3/7C5 cells with linearized plasmid pMCT-RUC ["B" cell lines] were grown to 100% confluence for measurements of light emission *in vivo* and *in vitro*. Light emission was
- 25 measured *in vivo* after about 30 generations as follows: growth medium was removed and replaced by 1 mL RPMI 1640 containing coelenterazine [1 mmol/L final concentration]. Light emission from cells was then visualized by placing the Petri dishes in a low light video image analyzer [Hamamatsu Argus-100]. An image was formed after 5 min. of photon

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accumulation using 100% sensitivity of the photon counting tube. For measuring light emission *in vitro*, cells were trypsinized and harvested from one Petri dish, pelleted, resuspended in 1mL assay buffer [0.5 mol/L NaCl, 1 mmol/L EDTA, 0.1 mol/L potassium phosphate, pH 7.4] and 5 sonicated on ice for 10 s. Lysates were than assayed in a Turner TD-20e luminometer for 10 s after rapid injection of 0.5 mL of 1 mmol/L coelenterazine, and the average value of light emission was recorded as LU [1 LU = 1.6 x 10⁶ hu/s for this instrument].

Independent cell lines of EC3/7C5 cells transfected with linearized 10 plasmid pMCT-RUC showed different levels of *Renilla* luciferase activity. Similar differences in light emission were observed when measurements were performed on lysates of the same cell lines. This variation in light emission was probably due to a position effect resulting from the random integration of plasmid pMCT-RUC into the mouse genome, since 15 enrichment for site targeting of the luciferase gene was not performed in this experiment.

To obtain transfectant populations enriched in cells in which the luciferase gene had integrated into the minichromosome, transfected cells were grown in the presence of ganciclovir. This negative selection 20 medium selects against cells in which the added pMCT-RUC plasmid integrated into the host EC3/7C5 genome. This selection thereby enriches the surviving transfectant population with cells containing pMCT-RUC in the minichromosome. The cells surviving this selection were evaluated in luciferase assays which revealed a more uniform level 25 of luciferase expression. Additionally, the results of *in situ* hybridization assays indicated that the *Renilla* luciferase gene was contained in the minichromosome in these cells, which further indicates successful targeting of pMCT-RUC into the minichromosome.

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Plasmid pNEM-1, a variant of pMCT-RUC which also contains λ DNA to provide an extended region of homology to the minichromosome [see, other targeting vectors, below], was also used to transfect EC3/7C5 cells. Site-directed targeting of the *Renilla* luciferase gene and 5 the hygromycin-resistance gene in pNEM-1 to the minichromosome in the recipient EC3/7C5 cells was achieved. This was verified by DNA amplification analysis and by *in situ* hybridization. Additionally, luciferase gene expression was confirmed in luciferase assays of the transfectants.

E. Protein secretion targeting vectors

10 Isolation of heterologous proteins produced intracellularly in mammalian cell expression systems requires cell disruption under potentially harsh conditions and purification of the recombinant protein from cellular contaminants. The process of protein isolation may be greatly facilitated by secretion of the recombinantly produced protein into 15 the extracellular medium where there are fewer contaminants to remove during purification. Therefore, secretion targeting vectors have been constructed for use with the mammalian artificial chromosome system.

A useful model vector for demonstrating production and secretion of heterologous protein in mammalian cells contains DNA encoding a 20 readily detectable reporter protein fused to an efficient secretion signal that directs transport of the protein to the cell membrane and secretion of the protein from the cell. Vectors pLNCX-ILRUC and pLNCX-ILRUCλ, described below, are examples of such vectors. These vectors contain DNA encoding an interleukin-2 (IL2) signal peptide-Renilla reniformis 25 luciferase fusion protein. The IL-2 signal peptide [encoded by the sequence set forth in SEQ ID No. 9] directs secretion of the luciferase protein, to which it is linked, from mammalian cells. Upon secretion from the host mammalian cell, the IL-2 signal peptide is cleaved from the fusion protein to deliver mature, active, luciferase protein to the

extracellular medium. Successful production and secretion of this heterologous protein can be readily detected by performing luciferase assays which measure the light emitted upon exposure of the medium to the bioluminescent luciferin substrate of the luciferase enzyme.

- 5 Thus, this feature will be useful when artificial chromosomes are used for gene therapy. The presence of a functional artificial chromosome carrying an IL-Ruc fusion with the accompanying therapeutic genes will be readily monitored. Body fluids or tissues can be sampled and tested for luciferase expression by adding luciferin and appropriate cofactors
10 and observing the bioluminescence.

1. Construction of Protein Secretion Vector pLNCX-ILRUC
Vector pLNCX-ILRUC contains a human IL-2 signal peptide-R. reniformis fusion gene linked to the human cytomegalovirus (CMV) immediate early promoter for constitutive expression of the gene in mammalian cells. The
15 construct was prepared as follows.

- a. Preparation of the IL-2 signal sequence-encoding DNA
A 69-bp DNA fragment containing DNA encoding the human IL-2 signal peptide was obtained through nucleic acid amplification, using appropriate primers for IL-2, of an HEK 293 cell line [see, e.g., U.S.
20 Patent No. 4,518,584 for an IL-2 encoding DNA; see, also SEQ ID No. 9; the IL-2 gene and corresponding amino acid sequence is also provided in the Genbank Sequence Database as accession nos. K02056 and J00264]. The signal peptide includes the first 20 amino acids shown in the translations provided in both of these Genbank entries and in SEQ ID NO. 9. The corresponding nucleotide sequence encoding the first 20 amino acids is also provided in these entries [see, e.g., nucleotides 293-52 of accession no. K02056 and nucleotides 478-537 of accession no. J00264], as well as in SEQ ID NO. 9. The amplification primers included an EcoRI site [GAATTC] for subcloning of the DNA fragment after

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ligation into pGEMT [Promega]. The forward primer is set forth in SEQ ID No. 11 and the sequence of the reverse primer is set forth in SEQ ID No. 12.

TTTGAATTCATGTACAGGATGCAACTCCTG forward [SEQ ID No. 11]

5 TTTGAATTAGGTGCAGTGTGAC reverse [SEQ ID No. 12]

b. Preparation of the R. reniformis luciferase-encoding DNA

The initial source of the R. reniformis luciferase gene was plasmid pLXSN-RUC. Vector pLXSN [see, e.g., U.S. Patent Nos.

- 10 5,324,655, 5,470,730, 5,468,634, 5,358,866 and Miller *et al.* (1989) Biotechniques 7:980] is a retroviral vector capable of expressing heterologous DNA under the transcriptional control of the retroviral LTR; it also contains the neomycin-resistance gene operatively linked for expression to the SV40 early region promoter. The R. reniformis 15 luciferase gene was obtained from plasmid pTZrLuc-1 [see, e.g., U.S. Patent No. 5,292,658; see also the Genbank Sequence Database accession no. M63501; and see also Lorenz *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:4438-4442] and is shown as SEQ ID NO. 10. The 0.97 kb EcoRI/SmaI fragment of pTZrLuc-1 contains the coding region of 20 the *Renilla* luciferase-encoding DNA. Vector pLXSN was digested with and ligated with the luciferase gene contained on a pLXSN-RUC, which contains the luciferase gene located operably linked to the viral LTR and upstream of the SV40 promoter, which directs expression of the neomycin-resistance gene.

25 c. Fusion of DNA encoding the IL-2 Signal Peptide and the R. reniformis Luciferase Gene to Yield pLXSN-ILRUC

- The pGEMT vector containing the IL-2 signal peptide-encoding DNA described in 1.a. above was digested with EcoRI, and the resulting 30 fragment encoding the signal peptide was ligated to EcoRI-digested

pLXSN-RUC. The resulting plasmid, called pLXSN-ILRUC, contains the IL-2 signal peptide-encoding DNA located immediately upstream of the R. reniformis gene in pLXSN-RUC. Plasmid pLXSN-ILRUC was then used as a template for nucleic acid amplification of the fusion gene in order to

5 add a SmaI site at the 3' end of the fusion gene. The amplification product was subcloned into linearized [EcoRI/SmaI-digested] pGEMT [Promega] to generate ILRUC-pGEMT.

10 d. **Introduction of the Fusion Gene into a Vector Containing Control Elements for Expression in Mammalian Cells**

Plasmid ILRUC-pGEMT was digested with KspI and SmaI to release a fragment containing the IL-2 signal peptide-luciferase fusion gene which was ligated to HpaI-digested pLNCX. Vector pLNCX [see, e.g., U.S. Patent Nos. 5,324,655 and 5,457,182; see, also Miller and

15 Rosman (1989) Biotechniques 7:980-990] is a retroviral vector for expressing heterologous DNA under the control of the CMV promoter; it also contains the neomycin-resistance gene under the transcriptional control of a viral promoter. The vector resulting from the ligation reaction was designated pLNCX-ILRUC. Vector pLNCX-ILRUC contains

20 the IL-2 signal peptide-luciferase fusion gene located immediately downstream of the CMV promoter and upstream of the viral 3' LTR and polyadenylation signal in pLNCX. This arrangement provides for expression of the fusion gene under the control of the CMV promoter. Placement of the heterologous protein-encoding DNA [i.e., the luciferase

25 gene] in operative linkage with the IL-2 signal peptide-encoding DNA provides for expression of the fusion in mammalian cells transfected with the vector such that the heterologous protein is secreted from the host cell into the extracellular medium.

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2. Construction of Protein Secretion Targeting Vector pLNCX-ILRUC λ

Vector pLNCX-ILRUC may be modified so that it can be used to introduce the IL-2 signal peptide-luciferase fusion gene into a mammalian artificial chromosome in a host cell. To facilitate specific incorporation of the pLNCX-ILRUC expression vector into a mammalian artificial chromosome, nucleic acid sequences that are homologous to nucleotides present in the artificial chromosome are added to the vector to permit site directed recombination.

10 Exemplary artificial chromosomes described herein contain λ phage DNA. Therefore, protein secretion targeting vector pLNCX-ILRUC λ was prepared by addition of λ phage DNA [from Charon 4A arms] to produce the secretion vector pLNCX-ILRUC.

15 3. Expression and Secretion of R. reniformis Luciferase from Mammalian Cells

a. Expression of R. reniformis Luciferase Using pLNCX-ILRUC

Mammalian cells [LMTK⁺ from the ATCC] were transiently transfected with vector pLNCX-ILRUC [$\sim 10 \mu\text{g}$] by electroporation [BIORAD, performed according to the manufacturer's instructions]. Stable transfectants produced by growth in G418 for neo selection have also been prepared.

Transfectants were grown and then analyzed for expression of luciferase. To determine whether active luciferase was secreted from the 25 transfected cells, culture media were assayed for luciferase by addition of coelentrazine [see, e.g., Matthews *et al.* (1977) Biochemistry 16:85-91].

The results of these assays establish that vector pLNCX-ILRUC is capable of providing constitutive expression of heterologous DNA in 30 mammalian host cells. Furthermore, the results demonstrate that the

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human IL-2 signal peptide is capable of directing secretion of proteins fused to the C-terminus of the peptide. Additionally, these data demonstrate that the R. reniformis luciferase protein is a highly effective reporter molecule, which is stable in a mammalian cell environment, and

5 forms the basis of a sensitive, facile assay for gene expression.

b. *Renilla reniformis* luciferase appears to be secreted from LMTK⁺ cells.

(i) *Renilla* luciferase assay of cell pellets

The following cells were tested:

- 10 cells with no vector: LMTK⁺ cells without vector as a negative control;
- cells transfected with pLNCX only;
- cells transfected with RUC-pLNCX [*Renilla* luciferase gene in pLNCX vector];
- 15 cells transfected with pLNCX-ILRUC [vector containing the IL-2 leader sequence + *Renilla* luciferase fusion gene in pLNCX vector].
- Forty-eight hours after electroporation, the cells and culture medium were collected. The cell pellet from 4 plates of cells was resuspended in 1 ml assay buffer and was lysed by sonication. Two
- 20 hundred μ l of the resuspended cell pellet was used for each assay for luciferase activity [see, e.g., Matthews *et al.* (1977) *Biochemistry* **16**:85-91]. The assay was repeated three times and the average bioluminescence measurement was obtained.

The results showed that there was relatively low background

25 bioluminescence in the cells transformed with pLNCX or the negative control cells; there was a low level observed in the cell pellet from cells containing the vector with the IL-2 leader sequence-luciferase gene fusion and more than 5000 RLU in the sample from cells containing RUC-pLNCX.

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(ii) **Renilla luciferase assay of cell medium**

Forty milliliters of medium from 4 plates of cells were harvested and spun down. Two hundred microliters of medium was used for each luciferase activity assay. The assay was repeated several times and the
5 average bioluminescence measurement was obtained. These results showed that a relatively high level of bioluminescence was detected in the cell medium from cells transformed with pLNCX-ILRUC; about 10-fold lower levels [slightly above the background levels in medium from cells with no vector or transfected with pLNCX only] was detected in the cells
10 transfected with RUC-pLNCX.

(iii) **conclusions**

The results of these experiments demonstrated that Renilla luciferase appears to be secreted from LMTK⁺ cells under the direction of the IL-2 signal peptide. The medium from cells transfected with Renilla
15 luciferase-encoding DNA linked to the DNA encoding the IL-2 secretion signal had substantially higher levels of Renilla luciferase activity than controls or cells containing luciferase-encoding DNA without the signal peptide-encoding DNA. Also, the differences between the controls and cells containing luciferase encoding-DNA demonstrate that the luciferase
20 activity is specifically from luciferase, not from a non-specific reaction. In addition, the results from the medium of RUC-pLNCX transfected cells, which is similar to background, show that the luciferase activity in the medium does not come from cell lysis, but from secreted luciferase.

25 c. **Expression of R. reniformis Luciferase Using pLNCX-ILRUCA**

To express the IL-2 signal peptide-R. reniformis fusion gene from an mammalian artificial chromosome, vector pLNCX-ILRUCA is targeted for site-specific integration into a mammalian artificial chromosome through homologous recombination of the λ DNA sequences contained in

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- the chromosome and the vector. This is accomplished by introduction of pLNCX-ILRUC λ into either a fusion cell line harboring mammalian artificial chromosomes or mammalian host cells that contain mammalian artificial chromosomes. If the vector is introduced into a fusion cell line harboring
- 5 the artificial chromosomes, for example through microinjection of the vector or transfection of the fusion cell line with the vector, the cells are then grown under selective conditions. The artificial chromosomes, which have incorporated vector pLNCX-ILRUC λ , are isolated from the surviving cells, using purification procedures as described above, and
- 10 then injected into the mammalian host cells.

Alternatively, the mammalian host cells may first be injected with mammalian artificial chromosomes which have been isolated from a fusion cell line. The host cells are then transfected with vector pLNCX-ILRUC λ and grown.

- 15 The recombinant host cells are then assayed for luciferase expression as described above.

F. Other targeting vectors

- These vectors, which are based on vector pMCT-RUC, rely on positive and negative selection to insure insertion and selection for the
- 20 double recombinants. A single crossover results in incorporation of the DT-A, which kills the cell, double crossover recombinations delete the DT-1 gene.

1. Plasmid pNEM1 contains:

- DT-A: Diphtheria toxin gene (negative selectable marker)
- 25 Hyg: Hygromycin gene (positive selectable marker)
- ruc: Renilla luciferase gene (non-selectable marker)
- 1: LTR-MMTV promoter
- 2: TK promoter
- 3: CMV promoter

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MMR: Homology region (plasmid pAG60)

2. plasmid pNEM-2 and -3 are similar to pNEM 1 except for different negative selectable markers:

pNEM-1: diphtheria toxin gene as "—" selectable marker

5 pNEM-2: hygromycin antisense gene as "—" selectable marker

pNEM-3: thymidine kinase HSV-1 gene as "—" selectable marker

3. Plasmid - λ DNA based homology:

pNEM λ -1: base vector

pNEM λ -2: base vector containing p5 = gene

10 1: LTR MMTV promoter

2: SV40 promoter

3: CMV promoter

4: μ TIIA promoter (metallothionein gene promoter)

— homology region (plasmid pAG60)

15 λ L.A. and λ R.A. homology regions for λ left and right arms (λ gt-WES).

EXAMPLE 13

Microinjection of mammalian cells with plasmid DNA

These procedures will be used to microinject MACs into eukaryotic
20 cells, including mammalian and insect cells.

The microinjection technique is based on the use of small glass capillaries as a delivery system into cells and has been used for introduction of DNA fragments into nuclei [see, e.g., Chalfie et al. (1994) Science 263:802-804]. It allows the transfer of almost any type of
25 molecules, e.g., hormones, proteins, DNA and RNA, into either the cytoplasm or nuclei of recipient cells. This technique has no cell type restriction and is more efficient than other methods, including Ca^{2+} -mediated gene transfer and liposome-mediated gene transfer. About 20-30% of the injected cells become successfully transformed.

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Microinjection is performed under a phase-contrast microscope. A glass microcapillary, prefilled with the DNA sample, is directed into a cell to be injected with the aid of a micromanipulator. An appropriate sample volume (1-10 μ l) is transferred into the cell by gentle air pressure exerted
5 by a transjector connected to the capillary. Recipient cells are grown on glass slides imprinted with numbered squares for convenient localization of the injected cells.

a. Materials and equipment

Nunclon tissue culture dishes 35 x 10 mm, mouse cell line EC3/7C5
10 Plasmid DNA pCH110 [Pharmacia], Purified Green Fluorescent Protein (GFP) [GFPs from *Aequorea* and *Renilla* have been purified and also DNA encoding GFPs has been cloned; see, e.g., Prasher *et al.* (1992) *Gene* 111:229-233; International PCT Application No. WO 95/07463, which is based on U.S. application Serial No. 08/119,678 and U.S. application
15 Serial No. 08/192,274], ZEISS Axiovert 100 microscope, Eppendorf transjector 5246, Eppendorf micromanipulator 5171, Eppendorf Cellolocate coverslips, Eppendorf microloaders, Eppendorf femtotips and other standard equipment

b. Protocol for injecting

20 (1) Fibroblast cells are grown in 35 mm tissue culture dishes (37° C, 5% CO₂) until the cell density reaches 80% confluence. The dishes are removed from the incubator and medium is added to about a 5 mm depth.

(2) The dish is placed onto the dish holder
25 and the cells observed with 10 x objective; the focus is desirably above the cell surface.

(3) Plasmid or chromosomal DNA solution [1 ng/ μ l] and GFP protein solution are further purified by centrifuging the

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DNA sample at a force sufficient to remove any particular debris [typically about 10,000 rpm for 10 minutes in a microcentrifuge].

(4) Two 2 μ l of the DNA solution (1 ng/ μ l) is loaded into a microcapillary with an Eppendorf microloader. During 5 loading, the loader is inserted to the tip end of the microcapillary. GFP (1 mg/ml) is loaded with the same procedure.

(5) The protecting sheath is removed from the microcapillary and the microcapillary is fixed onto the capillary holder connected with the micromanipulator.

10 (6) The capillary tip is lowered to the surface of the medium and is focussed on the cells gradually until the tip of the capillary reaches the surface of a cell. The capillary is lowered further so that the it is inserted into the cell. Various parameters, such as the level of the capillary, the time and pressure, are determined for the particular 15 equipment. For example, using the fibroblast cell line C5 and the above-noted equipment, the best conditions are: injection time 0.4 second, pressure 80 psi. DNA can then be automatically injected into the nuclei of the cells.

(7) After injection, the cells are returned to 20 the incubator, and incubated for about 18-24 hours.

(8) After incubation the number of 25 transformants can be determined by a suitable method, which depends upon the selection marker. For example, if green fluorescent protein is used, the assay can be performed using UV light source and fluorescent filter set at 0-24 hours after injection. If β -gal-containing DNA, such as DNA-derived from pHG110, has been injected, then the transformants can be assayed for β -gal.

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(c) **Detection of β -galactosidase in cells injected with plasmid DNA**

- The medium is removed from the culture plate and the cells are fixed by addition of 5 ml of fixation Solution I: (1% glutaraldehyde; 0.1
5 M sodium phosphate buffer, pH 7.0; 1 mM MgCl₂), and incubated for 15 minutes at 37° C. Fixation Solution I is replaced with 5 ml of X-gal
Solution II: [0.2% X-gal, 10 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆H₂O, 3.3 mM K₃Fe(CN)₆], and the plates are incubated for 30-60 minutes at 37° C. The X-gal solution
10 is removed and 2 ml of 70% glycerol is added to each dish. Blue stained cells are identified under a light microscope.

This method will be used to introduce a MAC, particularly the MAC with the anti-HIV megachromosome, to produce a mouse model for anti-HIV activity.

15

EXAMPLE 14

Transgenic (non-human) animals

- Transgenic (non-human) animals can be generated that express heterologous genes which confer desired traits, e.g., disease resistance, in the animals. A transgenic mouse is prepared to serve as a model of a
20 disease-resistant animal. Genes that encode vaccines or that encode therapeutic molecules can be introduced into embryos or ES cells to produce animals that express the gene product and thereby are resistant to or less susceptible to a particular disorder.

- The mammalian artificial megachromosome and others of the
25 artificial chromosomes, particularly the SATACs, can be used to generate transgenic (non-human) animals, including mammals and birds, that stably express genes conferring desired traits, such as genes conferring resistance to pathogenic viruses. The artificial chromosomes can also be

used to produce transgenic (non-human) animals, such as pigs, that can produce immunologically humanized organs for xenotransplantation.

For example, transgenic mice containing a transgene encoding an anti-HIV ribozyme provide a useful model for the development of stable

- 5 transgenic (non-human) animals using these methods. The artificial chromosomes can be used to produce transgenic (non-human) animals, particularly, cows, goats, mice, oxen, camels, pigs and sheep, that produce the proteins of interest in their milk; and to produce transgenic chickens and other egg-producing fowl, that produce therapeutic proteins
- 10 or other proteins of interest in their eggs. For example, use of mammary gland-specific promoters for expression of heterologous DNA in milk is known [see, e.g. U.S. Patent No. 4,873,316]. In particular, a milk-specific promoter or a promoter, preferably linked to a milk-specific signal peptide, specifically activated in mammary tissue is operatively
- 15 linked to the DNA of interest, thereby providing expression of that DNA sequence in milk.

- 1. Development of Control Transgenic Mice Expressing Anti-HIV Ribozyme**

- Control transgenic mice are generated in order to compare stability
- 20 and amounts of transgene expression in mice developed using transgene DNA carried on a vector (control mice) with expression in mice developed using transgenes carried in an artificial megachromosome.

- a. Development of Control Transgenic Mice Expressing β -galactosidase**

- 25 One set of control transgenic mice was generated by microinjection of mouse embryos with the β -galactosidase gene alone. The microinjection procedure used to introduce the plasmid DNA into the mouse embryos is as described in Example 13, but modified for use with embryos [see, e.g., Hogan *et al.* (1994) *Manipulating the Mouse Embryo*,
- 30 A :*Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY, see, especially pages 255-264 and Appendix 3]. Fertilized mouse embryos [Strain CB6 obtained from Charles River Co.] were injected with 1 ng of plasmid pCH110 (Pharmacia) which had been linearized by digestion with BamHI. This plasmid contains the β -galactosidase gene linked to the SV40 late promoter. The β -galactosidase gene product provides a readily detectable marker for successful transgene expression. Furthermore, these control mice provide confirmation of the microinjection procedure used to introduce the plasmid into the embryos. Additionally, because the mega-
5 chromosome that is transferred to the mouse embryos in the model system (see below) also contains the β -galactosidase gene, the control transgenic mice that have been generated by injection of pCH110 into embryos serve as an analogous system for comparison of heterologous gene expression from a plasmid versus from a gene carried on an artificial
10 chromosome.
15

After injection, the embryos are cultured in modified HTF medium under 5% CO₂ at 37°C for one day until they divide to form two cells. The two-cell embryos are then implanted into surrogate mother female mice [for procedures see, Manipulating the Mouse Embryo, A Laboratory Manual (1994) Hogan *et al.*, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 127 *et seq.*].

b. Development of Control Transgenic Mice Expressing Anti-HIV Ribozyme

One set of anti-HIV ribozyme gene-containing control transgenic mice was generated by microinjection of mouse embryos with plasmid pCEPUR-132 which contains three different genes: (1) DNA encoding an anti-HIV ribozyme, (2) the puromycin-resistance gene and (3) the hygromycin-resistance gene. Plasmid pCEPUR-132 was constructed by ligating portions of plasmid pCEP-132 containing the anti-HIV ribozyme
25

gene (referred to as ribozyme D by Chang *et al.* [(1990) Clin. Biotech. 2:23-31]; see also U.S. Patent No. 5,144,019 to Rossi *et al.*, particularly Figure 4 of the patent) and the hygromycin-resistance gene with a portion of plasmid pCEPUR containing the puromycin-resistance gene.

- 5 Plasmid pCEP-132 was constructed as follows. Vector pCEP4 (Invitrogen, San Diego, CA; see also Yates *et al.* (1985) Nature 313:812-815) was digested with Xhol which cleaves in the multiple cloning site region of the vector. This ~10.4-kb vector contains the hygromycin-resistance gene linked to the thymidine kinase gene promoter and
10 polyadenylation signal, as well as the ampicillin-resistance gene and ColE1 origin of replication and EBNA-1 (Epstein-Barr virus nuclear antigen) genes and OriP. The multiple cloning site is flanked by the cytomegalovirus promoter and SV40 polyadenylation signal.

- 15 Xhol-digested pCEP4 was ligated with a fragment obtained by digestion of plasmid 132 (see Example 4 for a description of this plasmid) with Xhol and Sall. This Xhol/Sall fragment contains the anti-HIV ribozyme gene linked at the 3' end to the SV40 polyadenylation signal. The plasmid resulting from this ligation was designated pCEP-132. Thus, in effect, pCEP-132 comprises pCEP4 with the anti-HIV ribozyme gene
20 and SV40 polyadenylation signal inserted in the multiple cloning site for CMV promoter-driven expression of the anti-HIV ribozyme gene.

- To generate pCEPUR-132, pCEP-132 was ligated with a fragment of pCEPUR. pCEPUR was prepared by ligating a 7.7-kb fragment generated upon Nhel/Nrul digestion of pCEP4 with a 1.1-kb Nhel/SnaBI fragment of pBabe [see Morgenstern and Land (1990) Nucleic Acids Res. 18:3587-3596 for a description of pBabe] that contains the puromycin-resistance gene linked at the 5' end to the SV40 promoter. Thus, pCEPUR is made up of the ampicillin-resistance and EBNA1 genes, as well as the ColE1 and OriP elements from pCEP4 and the puromycin-

resistance gene from pBabe. The puromycin-resistance gene in pCEPUR is flanked by the SV40 promoter (from pBabe) at the 5' end and the SV40 polyadenylation signal (from pCEP4) at the 3' end.

- Plasmid pCEPUR was digested with XbaI and SalI and the
- 5 fragment containing the puromycin-resistance gene linked at the 5' end to the SV40 promoter was ligated with XbaI-digested pCEP-132 to yield the ~12.1-kb plasmid designated pCEPUR-132. Thus, pCEPUR-132, in effect, comprises pCEP-132 with puromycin-resistance gene and SV40 promoter inserted at the XbaI site. The main elements of pCEPUR-132
- 10 are the hygromycin-resistance gene linked to the thymidine kinase promoter and polyadenylation signal, the anti-HIV ribozyme gene linked to the CMV promoter and SV40 polyadenylation signal, and the puromycin-resistance gene linked to the SV40 promoter and polyadenylation signal. The plasmid also contains the ampicillin-
- 15 resistance and EBNA1 genes and the ColE1 origin of replication and OriP.

- Zygotes were prepared from (C57BL/6JxCBA/J) F1 female mice [see, e.g., Manipulating the Mouse Embryo, A Laboratory Manual (1994) Hogan et al., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 429], which had been previously mated with a
- 20 (C57BL/6JxCBA/J) F1 male. The male pronuclei of these F2 zygotes were injected [see, Manipulating the Mouse Embryo, A Laboratory Manual (1994) Hogan et al., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY] with pCEPUR-132 (~3 µg/ml), which had been linearized by digestion with NruI. The injected eggs were then implanted
- 25 in surrogate mother female mice for development into transgenic offspring.

These primary carrier offspring were analyzed (as described below) for the presence of the transgene in DNA isolated from tail cells. Seven carrier mice that contained transgenes in their tail cells (but that may not

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carry the transgene in all their cells, i.e., they may be chimeric) were allowed to mate to produce non-chimeric or germ-line heterozygotes. The heterozygotes were, in turn, crossed to generate homozygote transgenic offspring.

5 2. **Development of Model Transgenic Mice Using Mammalian Artificial Chromosomes**

- Fertilized mouse embryos are microinjected (as described above) with megachromosomes (1-10 pL containing 0-1 chromosomes/pL) isolated from fusion cell line G3D5 or H1D3 (described above). The 10 megachromosomes are isolated as described herein. Megachromosomes isolated from either cell line carry the anti-HIV ribozyme (ribozyme D) gene as well as the hygromycin-resistance and β -galactosidase genes. The injected embryos are then developed into transgenic mice as described above.
- 15 Alternatively, the megachromosome-containing cell line G3D5^{*} or H1D3^{*} is fused with mouse embryonic stem cells [see, e.g., U.S. Patent No. 5,453,357, commercially available; see Manipulating the Mouse Embryo, A Laboratory Manual (1994) Hogan *et al.*, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pages 253-289]
- 20 following standard procedures see also, e.g., Guide to Techniques in Mouse Development in Methods in Enzymology Vol. 25, Wasserman and De Pamphilis, eds. (1993), pages 803-932]. (It is also possible to deliver isolated megachromosomes into embryonic stem cells using the Microcell procedure [such as that described above].) The stem cells are cultured in
- 25 the presence of a fibroblast [e.g., STO fibroblasts that are resistant to hygromycin and puromycin]. Cells of the resultant fusion cell line, which contains megachromosomes carrying the transgenes [i.e., anti-HIV ribozyme, hygromycin-resistance and β -galactosidase genes], are then transplanted into mouse blastocysts, which are in turn implanted into a

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surrogate mother female mouse where development into a transgenic mouse will occur.

Mice generated by this method are chimeric; the transgenes will be expressed in only certain areas of the mouse, e.g., the head, and thus 5 may not be expressed in all cells.

3. Analysis of Transgenic Mice for Transgene Expression

Beginning when the transgenic mice, generated as described above, are three-to-four weeks old, they can be analyzed for stable expression of the transgenes that were transferred into the embryos [or 10 fertilized eggs] from which they develop. The transgenic mice may be analyzed in several ways as follows.

a. Analysis of Cells Obtained from the Transgenic Mice

Cell samples [e.g., spleen, liver and kidney cells, lymphocytes, tail 15 cells] are obtained from the transgenic mice. Any cells may be tested for transgene expression. If, however, the mice are chimeras generated by microinjection of fertilized eggs or by fusion of embryonic stem cells with megachromosome-containing cells, only cells from areas of the mouse that carry the transgene are expected to express the transgene. If the 20 cells survive growth on hygromycin [or hygromycin and puromycin or neomycin, if the cells are obtained from mice generated by transfer of both antibiotic-resistance genes], this is one indication that they are stably expressing the transgenes. RNA isolated from the cells according to standard methods may also be analyzed by northern blot procedures 25 to determine if the cells express transcripts that hybridize to nucleic acid probes based on the antibiotic-resistance genes. Additionally, cells obtained from the transgenic mice may also be analyzed for β -galactosidase expression using standard assays for this marker enzyme [for example, by direct staining of the product of a reaction involving β -

galactosidase and the X-gal substrate, see, e.g., Jones (1986) EMBO 5:3133-3142, or by measurement of β -galactosidase activity, see, e.g., Miller (1972) in Experiments in Molecular Genetics pp. 352-355, Cold Spring Harbor Press]. Analysis of β -galactosidase expression is

- 5 particularly used to evaluate transgene expression in cells obtained from control transgenic mice in which the only transgene transferred into the embryo was the β -galactosidase gene.

Stable expression of the anti-HIV ribozyme gene in cells obtained from the transgenic mice may be evaluated in several ways. First, DNA 10 isolated from the cells according to standard procedures may be subjected to nucleic acid amplification using primers corresponding to the ribozyme gene sequence. If the gene is contained within the cells, an amplified product of pre-determined size is detected upon hybridization of the reaction mixture to a nucleic acid probe based on the ribozyme gene 15 sequence. Furthermore, DNA isolated from the cells may be analyzed using Southern blot methods for hybridization to such a nucleic acid probe. Second, RNA isolated from the cells may be subjected to northern blot hybridization to determine if the cells express RNA that hybridizes to nucleic acid probes based on the ribozyme gene. Third, the 20 cells may be analyzed for the presence of anti-HIV ribozyme activity as described, for example, in Chang et al. (1990) Clin. Biotech. 2:23-31. In this analysis, RNA isolated from the cells is mixed with radioactively labeled HIV gag target RNA which can be obtained by in vitro transcription of gag gene template under reaction conditions favorable to 25 in vitro cleavage of the gag target, such as those described in Chang et al. (1990) Clin. Biotech. 2:23-31. After the reaction has been stopped, the mixture is analyzed by gel electrophoresis to determine if cleavage products smaller in size than the whole template are detected; presence

of such cleavage fragments is indicative of the presence of stably expressed ribozyme.

b. Analysis of Whole Transgenic Mice

Whole transgenic mice that have been generated by transfer of the 5 anti-HIV ribozyme gene [as well as selection and marker genes] into embryos or fertilized eggs can additionally be analyzed for transgene expression by challenging the mice with infection with HIV. It is possible for mice to be infected with HIV upon intraperitoneal injection with high-producing HIV-infected U937 cells [see, e.g., Locardi *et al.* (1992) 10 *J. Virol.* 66:1649-1654]. Successful infection may be confirmed by analysis of DNA isolated from cells, such as peripheral blood mononuclear cells, obtained from transgenic mice that have been injected with HIV-infected human cells. The DNA of infected transgenic mice cells will contain HIV-specific gag and env sequences, as demonstrated 15 by, for example, nucleic acid amplification using HIV-specific primers. If the cells also stably express the anti-HIV ribozyme, then analysis of RNA extracts of the cells should reveal the smaller gag fragments arising by cleavage of the gag transcript by the ribozyme.

Additionally, the transgenic mice carrying the anti-HIV ribozyme 20 gene can be crossed with transgenic mice expressing human CD4 (i.e., the cellular receptor for HIV) [see Gillespie *et al.* (1993) *Mol. Cell. Biol.* 13:2952-2958; Hanna *et al.* (1994) *Mol. Cell. Biol.* 14:1084-1094; and Yeung *et al.* (1994) *J. Exp. Med.* 180:1911-1920, for a description of transgenic mice expressing human CD4]. The offspring of these crossed 25 transgenic mice expressing both the CD4 and anti-HIV ribozyme transgenes should be more resistant to infection [as a result of a reduction in the levels of active HIV in the cells] than mice expressing CD4 alone [without expressing anti-HIV ribozyme].

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4. Development of transgenic chickens using artificial chromosomes

- The development of transgenic chickens has many applications in the improvement of domestic poultry, an agricultural species of
- 5 commercial significance, such as disease resistance genes and genes encoding therapeutic proteins. It appears that efforts in the area of chicken transgenesis have been hampered due to difficulty in achieving stable expression of transgenes in chicken cells using conventional methods of gene transfer via random introduction into recipient cells.
- 10 Artificial chromosomes are, therefore, particularly useful in the development of transgenic chickens because they provide for stable maintenance of transgenes in host cells.
- a. Preparation of artificial chromosomes for introduction of transgenes into recipient chicken cells
- 15 (i) Mammalian artificial chromosomes
- Mammalian artificial chromosomes, such as the SATACs and minichromosomes described herein, can be modified to incorporate detectable reporter genes and/or transgenes of interest for use in developing transgenic chickens. Alternatively, chicken-specific artificial
- 20 chromosomes can be constructed using the methods herein. In particular, chicken artificial chromosomes [CACs] can be prepared using the methods herein for preparing MACs; or, as described above, the chicken libraries can be introduced into MACs provided herein and the resulting MACs introduced into chicken cells and those that are
- 25 functional in chicken cells selected.

As described in Examples 4 and 7, and elsewhere herein, artificial chromosome-containing mouse LMTK-derived cell lines, or minichromosome-containing cell lines, as well as hybrids thereof, can be transfected with selected DNA to generate MACs [or CACs] that have

integrated the foreign DNA for functional expression of heterologous genes contained within the DNA.

To generate MACs or CACs containing transgenes to be expressed in chicken cells, the MAC-containing cell lines may be transfected with

- 5 DNA that includes λ DNA and transgenes of interest operably linked to a promoter that is capable of driving expression of genes in chicken cells. Alternatively, the minichromosomes or MACs [or CACs], produced as described above, can be isolated and introduced into cells, followed by targeted integration of selected DNA. Vectors for targeted integration
10 are provided herein or can be constructed as described herein.

Promoters of interest include constitutive, inducible and tissue (or cell)-specific promoters known to those of skill in the art to promote expression of genes in chicken cells. For example, expression of the lacZ gene in chicken blastodermal cells and primary chicken fibroblasts has

- 15 been demonstrated using a mouse heat-shock protein 68 (*hsp 68*) promoter [*phspPTlacZpA*; see Brazolot *et al.* (1991) *Mol. Reprod. Devel.* 30:304-312], a Zn²⁺-inducible chicken metallothionein (cMt) promoter [*pCBcMtlacZ*; see Brazolot *et al.* (1991) *Mol. Reprod. Devel.* 30:304-312], the constitutive Rous sarcoma virus and chicken β -actin promoters
20 in tandem [*pmiwZ*; see Brazolot *et al.* (1991) *Mol. Reprod. Devel.* 30:304-312] and the constitutive cytomegalovirus (CMV) promoter. Of particular interest herein are egg-specific promoters that are derived from genes, such as ovalbumin and lysozyme, that are expressed in eggs.

- The choice of promoter will depend on a variety of factors,
25 including, for example, whether the transgene product is to be expressed throughout the transgenic chicken or restricted to certain locations, such as the egg. Cell-specific promoters functional in chickens include the steroid-responsive promoter of the egg ovalbumin protein-encoding gene [see Gaub *et al.* (1987) *EMBO J.* 6:2313-2320; Tora *et al.* (1988) *EMBO*

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J. Z:3771-3778; Park et al. (1995) Biochem. Mol. Biol. Int. (Australia)
36:811-816].

(ii) Chicken artificial chromosomes

- Additionally, chicken artificial chromosomes may be generated
- 5 using methods described herein. For example, chicken cells, such as primary chicken fibroblasts [see Brazolot et al. (1991) Mol. Reprod. Devel. 30:304-312], may be transfected with DNA that encodes a selectable marker [such as a protein that confers resistance to antibiotics] and that includes DNA (such as chicken satellite DNA) that
- 10 targets the introduced DNA to the pericentric region of the endogenous chicken chromosomes. Transfectants that survive growth on selection medium are then analyzed, using methods described herein, for the presence of artificial chromosomes, including minichromosomes, and particularly SATACs. An artificial chromosome-containing transfectant
- 15 cell line may then be transfected with DNA encoding the transgene of interest [fused to an appropriate promoter] along with DNA that targets the foreign DNA to the chicken artificial chromosome.

b. Introduction of artificial chromosomes carrying transgenes of interest into recipient chicken cells

- 20 Cell lines containing artificial chromosomes that harbor transgene(s) of interest (i.e., donor cells) may be fused with recipient chicken cells in order to transfer the chromosomes into the recipient cells. Alternatively, the artificial chromosomes may be isolated from the donor cells, for example, using methods described herein [see, e.g.,
- 25 Example 10], and directly introduced into recipient cells.

Exemplary chicken recipient cell lines include, but are not limited to, stage X blastoderm cells [see, e.g., Brazolot et al. (1991) Mol. Reprod. Dev. 30:304-312; Etches et al. (1993) Poultry Sci. 72:882-889;

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Petitte *et al.* (1990) Development 108:185-189] and chick zygotes [see, e.g., Love *et al.* (1994) Biotechnology 12:60-63].

For example, microcell fusion is one method for introduction of artificial chromosomes into avian cells [see, e.g., Dieken *et al.* [(1996)

- 5 Nature Genet. 12:174-182 for methods of fusing microcells with DT40 chicken pre-B cells]. In this method, microcells are prepared [for example, using procedures described in Example 1.A.5] from the artificial chromosome-containing cell lines and fused with chicken recipient cells.

Isolated artificial chromosomes may be directly introduced into
10 chicken recipient cell lines through, for example, lipid-mediated carrier systems, such as lipofection procedures [see, e.g., Brazolot *et al.* (1991) Mol. Reprod. Dev. 30:304-312] or direct microinjection. Microinjection is generally preferred for introduction of the artificial chromosomes into chicken zygotes [see, e.g., Love *et al.* (1994) Biotechnology 12:60-63].

15 c. Development of transgenic chickens

Transgenic chickens may be developed by injecting recipient Stage X blastoderm cells (which have received the artificial chromosomes) into embryos at a similar stage of development [see, e.g., Etches *et al.* (1993) Poultry Sci. 72:882-889; Petitte *et al.* (1990) Development 108:185-189; and Carsience *et al.* (1993) Development 117: 669-675].
20 The recipient chicken embryos within the shell are candled and allowed to hatch to yield a germline chimeric chicken that will express the transgene(s) in some of its cells.

Alternatively, the artificial chromosomes may be introduced into
25 chick zygotes, for example through direct microinjection [see, e.g., Love *et al.* (1994) Biotechnology 12:60-63], which thereby are incorporated into at least a portion of the cells in the chicken. Inclusion of a tissue-specific promoter, such as an egg-specific promoter, will ensure appropriate expression of operatively-linked heterologous DNA.

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The DNA of interest may also be introduced into a minichromosome, by methods provided herein. The minichromosome may either be one provided herein, or one generated in chicken cells using the methods herein. The heterologous DNA will be introduced
5 using a targeting vector, such as those provided herein, or constructed as provided herein.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the
10 appended claims.

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(ii) TITLE OF THE INVENTION: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

(iii) NUMBER OF SEQUENCES: 34

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 10-04-1997
- (C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/695,191
- (B) FILING DATE: 07-AUG-1996

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/682,080
- (B) FILING DATE: 15-JUL-1996
- (C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/629,822
- (B) FILING DATE: 10-APR-1996
- (C) CLASSIFICATION:

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- (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1293 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTCGTCAATT	TCAAGTCCTC	AAGTGGATGT	TTCTCATTTN	CCATGAATT	CAGTTTTCTN	180
GCCATATTCC	ACGTCCCTACA	GNGGACATTT	CTAAATTTC	CACCTTTTC	AGTTTCTCTC	240
GCCATATTTC	ACGTCCCTAAA	ATGTGTATTT	CTCGTTTNCC	GTGATTTCA	GTTTTCTCGC	300
CAGATTCCAG	GTCCTATAAT	GTGCATTCT	CATTNNCAC	GTTTTTCAGT	GATTTCGTCA	360
TTTTTCAG	TCGGCAAGTG	GATGTTCTC	ATTNCACATG	ATTNCAGTT	TTCTTGNAAT	420
ATTCCATGTC	CTACAATGAT	CATTTTAAT	TTTCCACCTT	TTCATTTTC	CACGCCATAT	480

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TTCATGTCCT	AAAGTGTATA	TTTCTCCCTT	TCCCGGATT	TCAGTTTCT	CGCCATATT	540
CAGGTCTAC	AGTGTGCATT	CCTCATT	CACCTTTTC	ACTGATT	TCATTTTCA	600
AGTCGTCAAC	TGGATCTTC	TAATTTCCA	TGATTTCA	TTATCTTGTC	ATATTCCATG	660
TCCTACAGTG	GACATTCTA	AATTTCCAA	CTTTTCAAT	TTTCTCGAC	ATATTGACG	720
TGCTAAAGTG	TGTATTTCTT	ATTTTCCGTG	ATTTTCAGTT	TTCTCGCCAT	ATTCCAGGTC	780
CTAATAGTGT	GCATTCTCA	TTTTTCACGT	TTTCAGTGA	TTTCGTCATT	TTTTCCAGTT	840
GTCAAGGGGA	TGTTTCTCAT	TTTCCATGAG	TGTCAGTTT	CTTGCTATAT	TCCATGTCCT	900
ACAGTGACAT	TTCTAAATAT	TATACCTTT	TCAGTTTTC	TCACCATATT	TCACGTCTA	960
AAGTATATAT	TTCTCATTTT	CCCTGATT	CCGTTTCTC	GCCATATTCC	AGGTCTACA	1020
GTGTGCATT	CTCATTTC	ACGTTTCA	GTAATTCTT	CATTTTTAA	GCCCTCAAAT	1080
GGATGTTCT	CATTTCAT	GATTTTCAGT	TTTCTTGCCA	TATACCATGT	CCTACAGTGG	1140
ACATTCTAA	ATTATCCACC	TTTTTCAGTT	TTTCATCGGC	ACATTTCACG	TCCTAAAGTG	1200
TGTATTCTA	ATTTTCAGTG	ATTTTCAGTT	TTCTCGCCAT	ATTCCAGGAC	CTACAGTGTG	1260
CATTTCAT	TTTCACGTT	TTTCAGTGA	TTC			1293

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1044 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TTTGAAACA	CTCTTTTGT	AAAATCTGCA	AGAGGATATT	TGGATAGCTT	TGAGGATTT	180
CGTTGGAAAC	GGGATTGTCT	TCATATAAAC	CCTAGACAGA	AGCATTCTCA	GAAGCTTCAT	240
TGGGATGTTT	CAGTTGAAGT	CACAGTGTG	AACAGTCCC	TTTCATAGAG	CAGGTTTGAA	300
ACACTCTTT	TTGTTAGTATC	TGGAAGTGG	CATTGGAGC	GATTCAGGA	CTGCGGTGAA	360
AAAGGAAATA	TCTTCCAATA	AAAGCTAGAT	AGAGGCAATG	TCAGAAACCT	TTTCATGAT	420
GTATCTACTC	AGCTAACAGA	GTGAACTT	CTTTTGAGAG	AGCAGTTTG	AAACACTCTT	480
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TCACAGAGTT	GAACATTCCC	TTTCATAGAG	CAGGTTGAA	ACACACTTTT	TGATGTATCT	660
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AACTAGACAG	AAGCATTCTC	AGAAA	TTGTGATG	CGCCCTCAAC	TAACAGTGT	780
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ATTTGTCTAG	CTTGAGGAT	TTCTTGGAA	ACGGGATTAC	ATATAAAAAG	CAGACAGCAG	900
CATTCCCAGA	ATCTTGT	TGATGTTGC	ATTCAAGTCA	CAGAGTTGAA	CATTCCCTT	960
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CAGGGGGGAT	CCTCTAGAA	TCCT				1044

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2492 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-198-

(ii) MOLECULE TYPE: Genomic DNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:
 (ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GGATCTATGG	GGGTGGGGAG	AAGCCCAGTG	ACAGTGCCTA	GAAGAGACAA	GGTGGCTGA	180
GAGGGTCTGA	GGAACATAGA	GCTGGCCATG	TTGGGGCCAG	GTCTCAAGCA	GGAAAGTGAGG	240
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ATTACAATGG	ACACAGGAGG	TTGGGACACC	TGGAGTCACC	AAACAAAACC	ATGCCAAGAG	420
AGACCATGAG	TAGGGTGTG	CAGTCCAGCC	CTCTGACTGA	GCTGCATTGT	TCAAATCCAA	480
AGGGCCCTG	CTGCCACCTA	GTGGCTGATG	GCATCCACAT	GACCCCTGGG	CACACCGT	540
TAGGGTCTCT	GTGAAGACCA	AGATCCTTGT	TACATTGAAC	GACTCCTAA	TGAGCAGAGA	600
TTTCCACCTA	TCGAAACAA	TCACATAAAA	TCCATCCTGG	AAAAAGCCTG	GGGGATGGCA	660
CTAAGGCTAG	GGATAGGGTG	GGATGAAGAT	TATAGTTACA	GTAAGGGGTT	TAGGGTTAGG	720
GATCAACGTT	GGTTAGGAGT	TAGGGATACA	GTAGGGTACC	GGTAGGGTTA	GGGGTTAGGG	780
TTAGGGGTTA	GGGTTAGGGT	TAGGGTTAGG	GTAGGGTTA	GGGGTTAGGG	GTAGGGTTA	840
GGGTTAGGGT	TTGGGGTGGC	GTATTTGGT	CTTATACGCT	GTGTTCCACT	GGCAATGAAA	900
AGAGTTCTG	TTTTCCCTC	AGCAATTGT	CATTTTAAA	AGAGTTAGC	AATTCTAAC	960
GATATAGACC	AGCTGTGCTA	TCTCATGTC	GTTCATCAATT	GTAACCACAT	TGTGGTTCA	1020
ATGTGTTTAC	TTGCCATCTG	TAGATCTTCT	TTGCGTGAGG	TGTCTGTTCA	GATGTGTGTG	1080
CATTTCTTGN	NTTNNGGCTG	TTTAACCTAT	TGTTAGTTT	TAATAATT	TTATATATT	1140
GAAGACAAAT	CTTCTCAGA	TGTGTATTTG	CAAATATTTC	TTCAATATGA	GGCTTGCTTT	1200
TGTCTCTAAC	AAGGTCTCTT	CAGAGATAAC	TTAAATATAA	GAAATCCACA	CTGTCACITC	1260
TTTGTGTAT	ATCTACCTT	TGTGTCAATT	GTAAAATTTC	ATTACCAAAC	CCAAAGGCAG	1320
ATAGCTTTTC	TTCTATTGTT	TCTTCTAGAA	ATTGTATAG	TTTGCAATT	TTAGTGTAA	1380
GATGATTITG	AGTGATTATT	TGTGTAAAGTT	GTAAAGTTT	CGTCTATATC	CATATCATT	1440
CTTATGGTT	CCAATTAATC	GTTCCTCAC	TATTTTGGG	AAAGACACAG	GATAGTGGC	1500
TTTGTAGAG	TAGATAGGTA	GCTAGACATG	AAACAGGAGG	GGCCTCCTGG	AAAAGGGAAA	1560
GTCTGGGAAG	GCTCACCTGG	AGGACCACCA	AAAATTCA	TATTAGTAGC	ATCTCTAGT	1620
CTGGAGTGG	TGGGCACTTG	TCAATTGTGG	GTAGGAGGGA	AAAGAGGTCC	TATGCAGAAA	1680
GAAACTCCCT	AGAACCTCTC	TGAAGATGCC	CCAATCATTC	ACTCTGCAAT	AAAATGTCA	1740
GAATATTGCT	AGCTACATGC	TGATAAGGNN	AAAGGGACAA	TTCTTAAGTG	AAACCTGGCA	1800
CCATAAGTAC	AGATTAGGGC	AGAGAAGGAC	ATTCAAAGA	GGCAGGCGCA	GTAGGTACAA	1860
ACGTGATCGC	TGTCAGTGTG	CCTGGGATGG	CGGGAAAGGAG	GCTGGTGC	GAGTGGATT	1920
GTATTGATCA	CCACACATAT	ACCTCAACCA	ACAGTGAGGA	GGTCCCACAA	GCCTAAGTGG	1980
GGCAAGTTGG	GGAGCTAAGG	CAGTAGCAGG	AAAACCAGAC	AAAGAAAACA	GGTGGAGACT	2040
TGAGACAGAG	GCAGGAATGT	GAAGAAATCC	AAAATAAAAT	TCCCTGCACA	GGACTCTTAG	2100
GCTGTTTAAT	GCATCGCTCA	GTCCCACCTC	TCCCTATT	TCTACAATAA	ACTCTTTACA	2160
CTGTGTTCT	TTTCAATGAA	GTATCTGCC	ATCTTGTAT	TGCCTCTTGG	TGAAAATGTT	2220
TCTTCCAAGT	TAACAAAGAA	CTGGGACATC	AGCTCTCCCC	AGTAATAGCT	CCGTTTCAGT	2280
TTGAATTTCAC	AGAACTGATG	GGCTTAATAA	CTGGCGCTCT	GACTTTAGTG	GTGCAGGAGG	2340
CCGTACACACC	GGGACCAAGA	GTGCCCTGCC	TAGTCCCCAT	CTGCCCGCAG	GTGGCGGGCTG	2400
CCTCGACACT	GACAGCAATA	GGGTCCGGCA	GTGTCCCCAG	CTGCCAGCAG	GGGGCGTACG	2460
ACGACTACAC	TGTGAGCAAG	AGGGCCCTGC	AG			2492

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

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(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGAATTCA TTGGGATGTT TCAGTTGA

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGAAAGTCCC CCCTAGGAGA TCTTAAGGA

29

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGCTTAATA CTCTGATGAG TCCGTGAGGA CGAAACGCTC TCGCACC

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO

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(v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:
 (ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGATTAAAT TAATTAAGCC CGGGC

25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:
 (ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TAAATTTAAT TAATTCGGGC CCGTCGA

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 69 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
 (D) OTHER INFORMATION IL-2 signal sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG TAC AGG ATG CAA CTC CTG TCT TGC ATT GCA CTA AGT CTT GCA CTT
 Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu

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GTC ACA AAC AGT GCA CCT ACT
 Val Thr Asn Ser Ala Pro Thr

69

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 945 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

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(A) NAME/KEY: Coding Sequence

(B) LOCATION: 1...942

(D) OTHER INFORMATION: Renilla Reiniformis Luciferase

(x) PUBLICATION INFORMATION:

PATENT NO.: 5,418,155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGC TTA AAG ATG ACT TCG AAA GTT TAT GAT CCA GAA CAA AGG AAA CGG Ser Leu Lys Met Thr Ser Lys Val Tyr Asp Pro Glu Gln Arg Lys Arg 1 5 10 15	48
ATG ATA ACT GGT CCG CAG TGG TGG GCC AGA TGT AAA CAA ATG AAT GTT Met Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val 20 25 30	96
CTT GAT TCA TTT ATT AAT TAT TAT GAT TCA GAA AAA CAT GCA GAA AAT Leu Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn 35 40 45	144
GCT GTT ATT TTT TTA CAT GGT AAC GCG GCC TCT TCT TAT TTA TGG CGA Ala Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg 50 55 60	192
CAT GTT GTG CCA CAT ATT GAG CCA GTA GCG CGG TGT ATT ATA CCA GAT His Val Val Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp 65 70 75 80	240
CTT ATT GGT ATG GGC AAA TCA GGC AAA TCT GGT AAT GGT TCT TAT AGG Leu Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg 85 90 95	288
TTA CTT GAT CAT TAC AAA TAT CTT ACT GCA TGG TTG AAC TTC TTA ATT Leu Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Leu Asn Phe Leu Ile 100 105 110	336
TAC CAA AGA AGA TCA TTT TTT GTC GGC CAT GAT TGG GGT GCT TGT TTG Tyr Gln Arg Arg Ser Phe Phe Val Gly His Asp Trp Gly Ala Cys Leu 115 120 125	384
GCA TTT CAT TAT AGC TAT GAG CAT CAA GAT AAG ATC AAA GCA ATA GTT Ala Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val 130 135 140	432
CAC GCT GAA AGT GTA GTA GAT GTG ATT GAA TCA TGG GAT GAA TGG CCT His Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro 145 150 155 160	480
GAT ATT GAA GAA GAT ATT GCG TTG ATC AAA TCT GAA GAA GGA GAA AAA Asp Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys 165 170 175	528
ATG GTT TTG GAG AAT AAC TTC TTC GTG GAA ACC ATG TTG CCA TCA AAA Met Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys 180 185 190	576
ATC ATG AGA AAG TTA GAA CCA GAA GAA TTT GCA GCA TAT CTT GAA CCA Ile Met Arg Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro 195 200 205	624

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TTC AAA GAG AAA GGT GAA GTT CGT CGT CCA ACA TTA TCA TGG CCT CGT Phe Lys Glu Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg 210 215 220	672
GAA ATC CCG TTA GTA AAA GGT GGT AAA CCT GAC GTT GTA CAA ATT GTT Glu Ile Pro Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val 225 230 235 240	720
AGG AAT TAT AAT GCT TAT CTA CGT GCA AGT GAT GAT TTA CCA AAA ATG Arg Asn Tyr Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met 245 250 255	768
TTT ATT GAA TCG GAT CCA GGA TTC TTT TCC AAT GCT ATT GTT GAA GGC Phe Ile Glu Ser Asp Pro Gly Phe Ser Asn Ala Ile Val Glu Gly 260 265 270	816
GCC AAG AAG TTT CCT AAT ACT GAA TTT GTC AAA GTA AAA GGT CTT CAT Ala Lys Lys Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His 275 280 285	864
TTT TCG CAA GAA GAT GCA CCT GAT GAA ATG GGA AAA TAT ATC AAA TCG Phe Ser Gln Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser 290 295 300	912
TTC GTT GAG CGA GTT CTC AAA AAT GAA CAA TAA Phe Val Glu Arg Val Leu Lys Asn Glu Gln 305 310	945

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTGAATTC A TGTACAGGAT GCAAATCCTG

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTGAATTCA GTAGGTGCAC TGTTTGTAC

30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1434 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: ; ;

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCTCCACGCA	CGTTGTGATA	TGTAGATGAT	AATCATTATC	AGAGCAGCGT	TGGGGGATAAA	60
TGTGACATT	TCCACTCCA	ATGACGGTGA	TGTATAATGC	TCAAGTATTTC	TCCTGCTTTT	120
TTACCACTAA	CTAGGAACGT	GGTTTGGCCT	TAATTCAAGAC	AGCCTTGGCT	CTGTCTGGAC	180
AGGTCCAGAC	GACTGACACC	ATTAACACTT	TGTCAGCCTC	AGTGACTACA	GTCATAGATG	240
AACAGGCCTC	AGCTAATGTC	AAGATACAGA	GAGGTCTCAT	GCTGGTTAAT	CAAATCATAG	300
ATCTTGTCCA	GATACAACTA	GATGTATTAT	GACAAATAAC	TCAGCAGGGA	TGTGAACAAA	360
AGTTTCCGGG	ATTGTGTGTT	ATTTCATTC	AGTATGTTAA	ATTTACTAGG	ACAGCTAATT	420
TGTCAAAAG	TCTTTTCAG	TATATGTTAC	AGAATTGGAT	GGCTGAATT	GAACAGATCC	480
TTCCGGAAATT	GAGACTTCAG	GTCAACTCCA	CGCGCTTGGG	CCTGTCGCTG	ACCAAAGGAT	540
TACCCAATTG	GATCTCTCA	GCATTTCTT	TCTTTAAAAA	ATGGGTGGGA	TTAATATTAT	600
TTGGAGATAC	ACTTTGCTGT	GGATTAGTGT	TGCCTCTTGT	ATTGGTCTGT	AAGCTTAAGG	660
CCCAAACATAG	GAGAGACAAG	GTGGTTATTG	CCCAGGCGCT	TGCAGGACTA	GAACATGGAG	720
CTTCCCCCTGA	TATATGGTTA	TCTATGCTTA	GGCAATAGGT	CGCTGCCAC	TCAGCTCTTA	780
TATCCCACGA	GGCTAGTCTC	ATTGTACGGG	ATAGAGTGAG	TGTGCTTCAG	CAGCCCGAGA	840
GAGTTGCAAG	GCTAACACT	GCAATGGAAA	GGCTCTGCGG	CATATATGTG	CCTATTCTAG	900
GGGGACATGT	CATCTTCAT	GAAGGTTCA	TGTCTTAGTT	CCCTCCCCC	AGGCAAAACG	960
ACACGGGAGC	AGGTCAAGGT	TGCTCTGGGT	AAAAGCCTGT	GAGCCTGGGA	GCTAATCCTG	1020
TACATGGCTC	CTTACCTAC	ACACTGGGGA	TTTGACCTCT	ATCTCCACTC	TCATTAATAT	1080
GGGTGGCCTA	TTTGCTCTTA	TTAAAAGGAA	AGGGGGAGAT	GTTGGGAGCC	GCGCCCACAT	1140
TCGCCGTTAC	AAGATGGGC	TGACAGCTGT	GTTCTAAGTG	GTAAACAAAT	AATCTGCGCA	1200
TGTGCCGAGG	GTGGTTCTTC	ACTCCATGTG	CTCTGCCCTTC	CCCGTGACGT	CAACTCGGCC	1260
GATGGGCTGC	AGCCAATCAG	GGAGTGACAC	GTCCTAGGCG	AAGGAGAATT	CTCCTTAATA	1320
GGGACGGGGT	TTCGTTCTCT	CTCTCTCTCT	TGCTTCTCTC	TCTTGCTTTT	TCGCTCTCTT	1380
GCTTCCCGTA	AAAGTATAAT	GATTATCATC	TACATATCAC	AACGTGCGTG	GAGG	1434

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1400 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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CCTCCACGCA	CGTTGTGATA	TGTAGATGAT	AATCATTATC	AGAGCAGCGT	TGGGGATAA	60
TGTCGACATT	TCCACTCCC	ATGACGGTGA	TGTATAATGC	TCAAGTATT	TCCTGTTTT	120
TTACCACTAA	CTAGGAAC	TG	GGTTGGCCT	TAATTCA	GACCTGGCT	180
AGGTCCAGAT	ACAAC	TAGAT	GTATTATGAC	AAATAACTCA	GCAGGGATGT	240
TTCCGGGATT	GGGTGTTATT	TCCATCCAGT	ATGTTAAATT	TACTAGGGCA	GCTAATTG	300
CAAAAAGTCT	TTTCCAGTAT	ATGTTACAGA	ATTGGATGGC	TGAATTGAA	CAGATCC	360
GGGAATTGAG	ACTTCAGGT	AACTCCACG	GCTTGACCT	GTCCTGACC	AAAGGATTAC	420
CCAATTGGAT	CTCCTCACG	CA	TTTTCTTCT	TTAAAAAATG	GGTGGGATTA	480
GAGATACACT	TTGCTGTG	GA	TTAGTGTG	TTCTTGATT	GGTCTGTAAG	540
AAACTAGGAG	AGACAAGG	TG	GTATTGCC	AGGCCTTGC	CTTAAGGCC	600
CCCCTGATAT	ATCTATG	CTT	AGGCAATAGG	TCGCTGGCA	AGGAGCTT	660
AGGCTAGTCT	CATTG	CACGG	GATAGAGTGA	GTGCTTCA	ATATCCATG	720
GGCTAACGAC	TGCAATGG	AA	AGGCTCTGCG	GCATATATGA	GCCTATTCTA	780
TCATCTTCA	AGAAGG	TG	GTGCTTCA	GGTAAACAAA	GGGAGACATG	840
CAGGTCAAGG	TTGCTCTG	GG	TAAAAGCCTG	TCAGCTTCT	GACACGGGAG	900
CCTTACCTA	CACACTGGG	AT	TTGACCTC	TATCTCCACT	GTACATGGCT	960
ATTTGCTT	ATTAAAAGG	AA	AGGGGGAGA	CTCATTAATA	TGGGTGGCCT	1020
CAAGATGGCG	CTGACAG	CTG	TGTTCTAAGT	TGTTGGGAGC	CGCGCCAC	1080
GGTGGTTCTT	CACTCCATG	TG	GCTCTG	GGTAAACAAA	TTCGCCGTTA	1140
CAGTCATCA	GGGAGTGA	CA	CGTCTAGGC	TCAGCTTCT	ATGCGCCGAG	1200
TTTCGTTTC	TCTCTCT	CT	GCTTCGCT	CGAGCAGCTG	1260	
TGTAAGAATA	AAGCTTG	CC	GCAGAAGATT	TTGCTCTG	1320	
TGAGAACCG	TCTAATAAC	AT	GGTGC	GTGTTCTTCC	1380	
TATCACAACG	TGCGTGG	GG	AAACCCGGGT	TGGCCGGTCG	1400	

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1369 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
 (iii) HYPOTHETICAL: NO
 (iv) ANTISENSE: NO
 (v) FRAGMENT TYPE:
 (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCTCCACGCA	CGTTGTGATA	TGTAGATGAT	AATCATTATC	ACTTTACGGG	TCCTTC	60
ACAAC	CGAGGCC	CG	TGCTCTG	ATAGATCTT	GCTGAAAAGG	120
ACACATTACT	CAAGGTGGG	TC	ATCTG	GTCAGATTCA	CACACACATG	180
ATTG	TGAA	AT	CAAAGTGA	GCTTAATATG	AATCTGCCA	240
TGGCATAATA	AA	ACACACA	AATTATCTAT	TATATAAAGG	TGCCCAC	300
ATACAAATAA	ATCATGGT	AG	ATAACAGTAA	GTGAGAG	ATGCTT	360
ACCTAATAA	AATTAAATT	AGAAGGTG	AA	TATACACTAC	AAAGTGA	420
AATT	TTTCTTA	AT	CCTTATA	AGTAGG	TTATTT	480
ATGTTCA	ATT	CTTCA	ATGAAAG	TTCTG	GCACATCC	540
TCAAATATG	TACACTT	GAT	TCCGTG	ATCTATC	ACAAGTTG	600
TTCCTTCG	ACTTG	ATT	ATTACAAG	TTATTAT	ATTGATTG	660
CTCTAACCAT	TTTATAAC	AT	CTTTAT	TCTCTCAG	CTTCATT	720
ATGCCAGAGA	GAAC	TTGAG	AGATAAG	AGAGTCAG	TGAGGCAC	780
GTTCCTTGG	AACTCAGG	AT	CAAATTCA	TTGTCAGG	ACCTCCTG	840
CAGTGCCTCC	ATCTTG	CC	CTGAAC	TGGCAGCAT	CACTTTTAC	900
GTGAAC	TG	AT	AGCAG	AGACAGAC	GCTACACT	960
ACTATTCTA	CTAGGTG	CT	ATGCA	GGT	CCATGC	1020
TTACATTG	TTCTATAG	CT	TAGTACT	TATTCG	TGAAAGAAAA	1080
CCTAGAC	CTTCA	TT	TTAGTACT	GAG	TTAATCAGG	1140
TACGGATG	TG	AA	AGTTCTACT	TA	TCCAAAACAG	1200

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CACTTATTGG	GAATGTTTT	GTTAATAAAA	GATTCAAGGTG	TTACTCTAGG	TCAAGAGAAT	1260
ATTAACATC	AGTCCCAAAT	TACAAACTTC	AATAAAAGAT	TTGACTCTCC	AGTGGTGGCA	1320
ATATAAGTG	ATAATGATTA	TCATCTACAT	ATCACAAACGT	CGGTGGAGG		1369

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22118 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAATTCCCT ATCCCTAAC	CAGATTGGTG	GAATAACTTG	GTATAGATGT	TTGTGCATTA	60
AAAACCTGT AGGATCTTCA	CTCTAGGTCA	CTGTTCAGCA	CTGGAACCTG	AATTGTGGCC	120
CTGAGTGT GGTCTGGGA	CATATGCAGT	TCTGCACAGA	CAGACAGACA	GACAGACAGA	180
CAGACAGACA GACAGACGTT	ACAAACAAAC	ACGTTGAGCC	GTGTGCCAAC	ACACACACAA	240
ACACCACTCT GGCCATAATT	ATTGAGGACG	TTGATTATT	ATTCTGTGTT	TGTGAGTCTG	300
TCGTCTGTC TGTCTGTCTG	TCTGTCTGTC	TATCAAACCA	AAAGAAACCA	ACAAATTATG	360
CCTGCCGCCC TGCCGCTG	CCTACACAGA	GAATGATTT	CTTCAATCAA	TCTAAAACGA	420
CCTCCTAAGT TTGCCCTTTT	TCTCTTCTT	TATCTTTTC	TTTTTCTTT	TCTCTTCTCT	480
TCCTCCCTTC CTTCCCTCCT	TCCTCCCTT	CTTCTTTCTT	TTCTTCTCTT	CTTACTTTCT	540
TTCTTCCCTT CTTACATTTA	TTCTTTCTAT	ACATAGTTTC	TTAGTGTAAAG	CATCCCTGAC	600
TGTCTTGAAG ACACATTGTA	GGCCCTAAC	CTGTAAGAGC	CTTCCCTCTG	TTTTCAATG	660
CTGGCATGAA TGTTGTACCT	CACTATGACC	AGCTTAGTCT	TCAAGTCTGA	TTTACTGGAA	720
AGGAGTTCGA AGAACAGCTG	TTATATTTTT	CATTATTAT	TGCATTTAA	TTAAAATTAA	780
ATTTCACCAA AAAGATTAG	ACTGACCAAT	TCAGAGTCTG	CCGTTAAAAA	GCATAAGGAA	840
AAAGTAGGAG AAAAACGTGA	GGCTGTCTGT	GGATGGTCGA	GGCTGCTTTA	GGGAGCCTCG	900
TCACCATTTCT GCACCTGAA	ACCGGGCCAC	TAGAACCGG	TGAAGGGAGA	ACCAAAAGCG	960
ACCTGGAAAC AATAGGTAC	ATGAAGGCCA	GCCACCTCCA	TCTTGTGTG	CGGGAGTTCA	1020
GTTAGCAGAC AAGATGGCTG	CCATGCACAT	GTTGTCTTTC	AGCTTGTGTA	GGTCAAAGTA	1080
CAACCGAGTC ACAGAACAAAG	GAAGTATACA	CAGTGTGTT	CAGGTAGCTC	AGAGTTTACA	1140
CAGAGAAACC ACATCTTGA	AAAAACAAAA	AAAAATAATA	AAAAATAATA	ATTTAAAAAT	1200
TTAAAAAATAG CGGGAGTGA	TGGCGCATGT	CTTAAATCCC	AGCTCTTTC	AGGCAGAGAT	1260
GGGAGGATT CTGAGTTGA	GGCCAGCCTG	GTCTGCAAAG	TGAGTCCAG	GACAGTCAGG	1320
GCTATACAGA GAAACCTGT	CTTGAAAACT	AAACTAAATT	AAACTAAACT	AAACTAAAAAA	1380
AAATATAAAAT AAAAATTATA	AAAGAATTATA	AAAAACTACA	GAAATCAAAC	ATAAGCCAC	1440
GAGATGGCAA GTAACGTGAA	TCATAGCAGA	AATATTATAC	ACACACACAC	ACACAGACTC	1500
TGTCATAAAA TCCAATGTG	CTTCATGATG	ATCAAATTTC	GATAGTCAGT	AAATACTAGAA	1560
GAATCATATG TCTGAAAATA	AAAGCCAGAA	CTTTTCTG	TTTTGTTTC	TTTGCCCCCA	1620
AGATAGGGTT TCTCTCAGTG	TATCCCTGGC	ATCCCTGCCT	GGAACCTTCT	TTGTAGGTTT	1680
GGTAGCCTCA AACTCAGAGA	GGTCCTCTC	GGCTGCTG	CTGCTGCGCT	GCCTGCCTGC	1740
CTGCTGCGCT GCCTGCCTCA	CTTCTCTG	CACCCACACA	ACCGAGTCGA	ACCTAGGATC	1800
TTTATTTCTT TCTCTTCTC	CTCTCTTCT	TTCTTCTTT	CTTCTTTCT	TTCTTTCTTT	1860
CTTTCTTCTT TTCTTATTCA	ATTAGTTTC	AATGTAAGTG	TGTGTTGTG	CTCTATCTGC	1920
TGCCTATAGG CCTGTTGCC	AGGAGAGGGC	AACAGAACCT	AGGAGAAACC	ACCATGCAGC	1980
TCCTGAGAAT AAGTAAAAAA	ACAACAAAAA	AAGGAAATT	TAATCACATA	GAATGTAGAT	2040
ATATGCCGAG GCTGTCAGAG	TGCTTTTAA	GGCTTAGTGT	AAGTAATGAA	AATTGTTGTG	2100
TGTCTTTAT CCAAACACAG	AAGAGAGGTG	GCTCGGCCTG	CATGTCTGTT	GTCTGCATGT	2160
AGACCAGGCT GGCCTTGAAC	ACATTAATCT	GTCTGCCTCT	GCTTCCCTAA	TGCTGCAGATT	2220
AAAGGCATGT GCCACCACTG	CCCGGACTGA	TTTCTTCTTT	TTTTTTTTTT	TGGAAAAATAC	2280
CTTTCTTCTT TTTTCTCTCT	CTCTTCTTC	CTCCTTCCT	TTCTTTCTAT	TCTTTTTTTC	2340
TTTCTTTTTT CTTTTTTTT	TTTTTTTAA	AATTTGCCTA	AGGTTAAAGG	TGTGCTCCAC	2400
AATTGCCTCA GCTCTGCTCT	AATTCTTTT	AAAAAAAAAC	AAACAAAAAA	AAAACAAAAA	2460

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CAGTATGTAT	GTATGTATAT	TTAGAAGAAA	TACTAATCCA	TTAATAACTC	TTTTTCCTA	2520
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TTCAAATTTC	TGTGTCAAG	GTCACCCCTGG	CTTACAAAGT	GAGTCCAAG	TCCGATAGGG	2640
CTACACAGAA	AAACCATATC	TCAGAAAAAA	AAAAGTCCC	AAACACACAC	ACACACACAC	2700
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AAGTCGTGCC	AAAATAAT	ATTTTCTGG	CCAAAGTGAA	AGCAAATCAC	TATGAAGAGG	2820
TACTCCTAGA	AAAATAAT	ACAAACGGGC	TTTTTAATCA	TTCCAGCACT	TTTTTAATT	2880
AACTCTGAAT	TTAGTCTTGG	AAAAGGGGGC	GGGTGTGGGT	GAGTGAAGGGC	GAGCGAGCAG	2940
ACGGGCGGGC	GGGCGGGTGA	GTGGCCGGCG	GCGGTGGCAG	CGAGCACCAG	AAAACAACAA	3000
ACCCCAAGCG	GTAGAGTGTGTT	TTAAAAATGAA	GACCTAAATG	TGGTGAACG	GAGGTGCGCG	3060
CCACCCCTCCT	CTTCCACTGTC	TTAGATGTC	CCTTCCCCCTT	ACTGTGCTCC	CTTCCCCCTAA	3120
CTGTGCCCTAA	CTGTGCCCTGT	TCCCTCACCC	CGCTGATTG	CCAGCGACGT	ACTTTGACTT	3180
CAAGAACGAT	TTTGCTCTGT	TTCACCGCTC	CCTGTCATAC	TTTCGTTTT	GGGTGCCCCGA	3240
GTCTAGCCCG	TTCGCTATGT	TCGGGGCGGGA	CGATGGGGAC	CGTTCTGCC	ACTCGGGAGA	3300
AGTGGTGGGT	GGGTACGCTG	CTCCGTCGTG	CCTGCGTGTG	TGCGGAACG	TGAGCTCGGG	3360
AGACCCCTCCG	GAGAGACAGA	ATGAGTGAAGT	GAATGTGGCG	GCGCGTACG	GATCTGTATT	3420
GGTTTGTATG	GTTGATCGAG	ACCATTGTCG	GGCGACACCT	AGTGGTGACA	AGTTTCGGGA	3480
ACGCTCCAGG	CCTCTCAGGT	TGGTGACACA	GGAGAGGGAA	GTGCGTGTGG	TGAGGCAGACC	3540
AGGGTGACAG	GAGGCCGGGC	AAGCAGGCGG	GAGCGTCTCG	GAGATGGGTG	CCTGTTTAAG	3600
GACGGTCTCT	AAACAAGGAGG	TCGTACAGGG	AGATGGCAA	AGCAGACCGA	TTGCTGTAC	3660
GCCCTTTTGG	GAAAATGCT	AGGGTTGGTG	GCAACGTTAC	TAGGTCGACC	AGAAGGCTTA	3720
AGTCTTACCC	CCCCCCCCCT	TTTTTTTTT	TTTCTCCAG	AAGCCCTCTC	TTGTCCTCCGT	3780
CACCGGGGGC	ACCGTACATC	TGAGGGCCAG	AGGACGCGAT	GGGCCCCGGCT	TCCAAGCCGG	3840
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GGGCCCCGGC	TTCCAAGCCG	GTGTGGCTCG	GCCAGCTGGA	GCTTGGGTC	TTTTTTTTT	4020
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TCTGAGGCCG	AGAGGACGCG	ATGGGTGGC	TTCCAAGCCG	ATGTGGCGGG	GCCAGCTGGA	4140
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TACTTCTGAG	GCCGAGAGGA	CGTGTATGGC	CCGGGTTCCA	GGCGGATGTC	GCCCCGGTCAG	4260
CTGGAGCTTT	GGATCTTTT	TTTTTTTTT	CCTCCAGAAG	CCCTCTCTTG	TCCCCGTAC	4320
CGGGGGCACC	TTACATCTGA	GGGCGAGAGG	ACGTGATGGG	TCCGGCTTCC	AAGCCGATGT	4380
GGGGGGGCCA	GCTGGAGCTT	CGGGTTTTT	TTTTTCTC	CAGAACCCCT	CTCTGTCTCC	4440
CGTCACCGGG	GGCGCTGTAC	TTCTGAGGCC	GAGAGGACGT	GATGGGGCCG	GTTCCAGGC	4500
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TCTGTCCCC	GTCACCGGGG	GCACCGTACA	TCTGAGGCCG	AGAGGACACG	ATGGGCCTGT	4620
CTTCAAGCC	GATGTGGCCC	GGCCAGCTGG	AGCTTCGGGT	CTTTTTTTT	TTTTTCTC	4680
CAGAACGCTT	GTCTGTCGCT	GTCACCCGGG	GCGCTGTACT	TCTGAGGCCG	AGAGGACGCG	4740
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CACCTTACAT	CTGAGGCCA	GAGGACACGA	TGGGCCCCGG	TTCCAGGCCG	ATGTGGCCCG	5040
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ACCGGTGGCA	CTGTACATCT	GAGGCGGAGA	GGACATTATG	GGCCCGGCTT	CCAATCCGAT	5160
GTGGCCCGGT	CAGCTGGAGC	TTTGGATCTT	ATTTTTTTT	TAATTTTTTC	TTCCAGAACG	5220
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GGGAAAGCTA	TGGGCGCGGT	TTTCTTTCAT	TGACCTGTG	GTCTTATCAG	TTCTCCGGGT	5460
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CACCGCTGTCC	TTTCCCTATT	AAACACTAAAG	GACACTATAA	AGAGACCCCT	TCGATTTAAG	5700
GCTGTTTGC	TTGTCCAGCC	TATTCTTTT	ACTGGCTTGG	GTCTGTCGCG	GTGCGTGAAG	5760
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GCAGCTTGTG	ACAACCTGGG	GCTGTGACTT	TGCTGCGTGT	CAGACGTTT	TCCCAGATTC	5880
CCCGAGGTGT	CGTTGTACACA	CCTGCCCCGG	TTGGAATGGT	GGAGCCAGCT	GTGGTTGAGG	5940
GCCACCTTAT	TTCGGCTCAC	TTTTTTTTT	TTTTTTTCTC	TTGGAGCTCC	GAACCTCCGC	6000
TCTTTCTCT	TCCCGGTCTT	TCTTCCACAT	GCCTCCCGAG	TGCATTCTT	TTGTTTTTT	6060
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CGTTGTGTT	TCTTGTGTTG	TGTCTGCCG	TATCAGTAAC	TGTCTGCC	CGCGTGTAA	6240
ACATTCTAT	CTCGCTTGTT	TCTCCCATT	GCGCGTCGTT	GCTCACTCTT	AGATCGATGT	6300
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CGCACTTTC	TCAGTGGTT	GCCTGGTCT	TGTTGGATGTG	TGAGGGCGCC	GGTTGTGCC	6540
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CCGGTGGCGT	TGCATACCCCT	TCCCCTGCTGG	TGTTGCCACG	CGCTGTTTCT	TGTAAGCGTC	6660
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GCGCTCCCCA	TTCCCTGGTG	TGCCCTCGGT	GCTCCGTCG	GCTGTGTGCC	TTCCCGTTTG	6780
TGTCTGAGAA	GGCCCGTGA	GGGGGGTCTGA	GGAGAGAAAGG	AGGGGCAAGA	CCCCCCTTCT	6840
TCGTGGGGT	AGGCGCCAC	CCCGCGACTA	GTACGCCCTGT	GCGTAGGGCT	GGTGTGAGC	6900
GGTCGCGGCT	GGGGTTGGAA	AGTTTCTCGA	GAGAATCTATT	GCTTTCCCGT	GGGGAGCTTT	6960
GAGAGGCCTG	GCTTCGGGG	GGGACCGGTT	GCAGGGTCTC	CCCTGTCCGC	GGATGCTCAG	7020
AATGCCCTTG	GAAGAGAAC	TTCCCTGTTG	CGCAGACCCC	CCCAGCGCGG	CGCCCGCGTG	7080
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GTCGGGGTTT	TGGGTCCGTC	CCGCCCTCAG	TGAGAAAGGT	TCTCTCTCTA	GCTATCTTCC	7200
GGAAAGGGTG	CGGGCTCTT	ACGGTCTCGA	GGGGTCTCTC	CCGAATGGTC	CCCTGGAGGG	7260
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GGCCTCCCCG	CTCCGAGTTC	GGGGAGGGAT	CACGCGGGGC	AGAGCCTGTC	TGTCGTCTG	7380
CCGTTGCTG	GGAGCATGTG	GCTCGGCTTG	TGTGGTTGGT	GGCTGGGGAG	AGGGCTCCGT	7440
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TCCGTCGCGT	GCCTCCCTCT	CGCTCGCGTC	CACGACTTTG	GCCGCTCCCG	CGACGGCGGC	7620
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CCCCCTTCCC	CGCGGCAGCG	TTCCCACGGC	TGCGAAATC	GCGGGAGTC	TCCCTCCCC	7740
CCTCGGGGTC	GAGAGGGTCC	GTGCTCTGGC	TTGATTGATC	TGCTCTCGG	GGACGGGACC	7800
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CCCCCTCCCC	GCTCGCCGCA	GCCGCTCTT	TTTCCTCTCT	CCCCCCCCTCT	CCTCTGACTG	8220
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CGGTCAACCGG	GGTCTTGGGG	GGGGGCCGAG	GGGTAAGAAA	GTCGGCTCGG	CGGGCGGGAG	8340
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CATCTCTCGC	GCAATGGCGC	CGCCCGAGTT	CACGGTGGGT	TGCTCTCTCG	CCTCCGCTTC	8820
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GAGCCCCCTG	CGCACCCGGCC	GGTGTGCGGT	TTCGCGCCGC	GGTCAGTTGG	GCCCTGGCGT	9060
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TCCGGCCGCA	TGCACTCTCC	CGTTCCGCGC	GAGCGCCCGC	CCGGCTCAC	CCCGGTTTGT	9540
CCTCCCGCGA	GGCTCTCCGC	CGCCGCCGCC	TCTCTCTCT	CTCTCGCGCT	CTCTGCTCCG	9600
CCTGGTCTCG	TCCCCCCCCC	GACGCTCGC	TCGCGCTTCC	TTACCTGGTT	GATCCTGCCA	9660
GGTAGCATA	GCTTGTCTCA	AAGATTAAGC	CATGCATGTC	TAAGTACGCA	CGGCCGGTAC	9720
AGTGAACACTG	CGAATGGCTC	ATTAATCAG	TTATGGTTCC	TTTGGTGTG	CGCTCCCTCTC	9780

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CTACTTGGAT	AACTGTGGTA	ATTCTAGAGC	TAATACATGC	CGACGGGCGC	TGACCCCCCT	9840
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GAGCCTGAGA	AACGGCTACC	ACATCCAAGG	AAGGCAGCAG	GCGCGCAAAT	TACCCACTCC	10140
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GATTCAACCC	GCGCGCGCGC	GTCCGGCCGT	GCCCGGGTGGT	CCCGGCGGAT	CTTTCGGCT	14280
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GGCGGTGCGC	CGCGACCGGC	TCCGGGACGG	CCGGGAAGGC	CCGGTGGGGA	AGGTGGCTCG	14520
GGGGGGGGCGG	CGCGTCTCAG	GGCGCGCGA	ACCACCTCAC	CCCGAGTGTG	ACAGCCCTCC	14580
GGCCCGCGCTT	TCGCCGAATC	CCGGGGCCGA	GGAAGGCCAGA	TACCCGTCGC	CGCGCTCTCC	14640
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GGCGCGACCG	CTCTCCACC	CCCCCTCGTC	GCTCTCTCG	GGGCCCCGGT	GGGGGCGGGG	14760
CGGACTGTCC	CCAGTGCGCC	CGGGCGCTCG	TCGGCGCCGT	GGGTCCCGGG	GGGACCGTCG	14820
GTCACGCGTC	TCCCCGACGA	GCGGAGCGA	CGGGGTGCGG	GGCGATGTG	GCTACCCACC	14880
CGACCCGCT	TGAAACACGG	ACCAAGGAGT	CTAACCGCGTG	CGCGAGTCAG	GGGCTCGTCC	14940
GAAAGCCGCC	GTGGCGCAAT	GAAGGTGAAG	GGCCCCGCC	GGGGGCCCGA	GGTGGGATCC	15000
CGAGGCCCT	CCAGTCCGCC	GAGGGCGCAC	CACCGGCCG	TCTCGCCCG	CGCGCCGGGG	15060
AGGTGGAGCA	CGAGCGTACG	CGTTAGGACC	CGAAAGATGG	TGAACATATGC	TTGGGCAGGG	15120
CGAAGCCAGA	GGAAACTCTG	GTGGAGGTCC	GTAGCGGTCC	TGACCGTCAA	ATCGGTGTC	15180
CGACCTGGGT	ATAGGGGCGA	AAGACTAATC	GAACCATCTA	GTAGCTGGTT	CCCTCCGAAG	15240
TTTCCCTCAG	GATACTGTC	GCTCTCGCTC	CCGACGATACG	CAGTTTATC	CGGTAAAGCG	15300
AATGATTAGA	GGTCTTGGGG	CCGAAACGAT	CTCAACCTAT	TCTCCTAACTT	TAATGGGTA	15360
AGAAGCCCGG	CTCGCTGGCG	TGGAACCGGG	CGTGAATG	GAGTGCCTAG	TGGGCCACTT	15420
TTGGTAAGCA	GAACTGGCGC	TGCGGGATGA	ACCGAACGCC	GGGTTAAGGC	GCCCAGATGCC	15480
GACGCTCATC	AGACCCCAGA	AAAGGTGTTG	GTTGATATAG	ACAGCAGGAC	GGTGGCCATG	15540
GAAGTCGGAA	TCCGCTAAGG	AGTGTGTAAC	AACTCACCTG	CCGAATCAAC	TAGCCCTGAA	15600
AATGGATGGC	GCTGGAGCGT	CGGGCCCAT	CCCAGGCCGT	GCCGAGTCG	GAACGGAACG	15660
GGACGGGAGC	GGCCCGGGGT	GCGCGTCTCT	CGGGGTGCGG	GGTGCCTGGC	GGGGGCCCGT	15720
CCCCCGCCTC	CCCTCCGCGC	GCCGGGTTCG	CCCCCGCGGC	GTCGGGCCCC	GGCGAGCCTA	15780
CGCCCGCGACG	AGTAGGAGGG	CCGCTCGGGT	GAGCCTTGA	GCCTAGGGCG	GGGGCCCCGG	15840
TGGAGCCGCC	GCAGGTGCGA	ATCTTGGTGG	TAGTAGCAA	TATCCTAAACG	AGAACTTTGA	15900
AGGCCGAAGT	GGAGAAGGGT	TCCATGTGAA	CAGCAGTTGA	ACATGGGTCA	GTCGGTCTCG	15960
AGAGATGGGC	GAGTGGCGTT	CCGAAGGGAC	GGCGATG	CTCCGTTGCC	CTCGGCCGAT	16020
CGAAAGGGAG	TGCGGTTCA	ATCCCCGAAT	CCGGAGTGGC	GGAGATGGGC	GGCGCGAGGC	16080
CAGTGCCTA	ACCGCGACCGA	TCCCGGAGAA	GCCGGCGGG	GGCCTCGGGG	AGAGTTCTCT	16140
TTTCTTGTG	AAGGGCAGGG	CGCCCTGGAA	TGGGTTCGCC	CCGAGAGAGG	GGCCCGTGCC	16200
TTGGAAAGCG	TCGCGGTTCC	GGCGGGCTCC	GGTGAGCTCT	CGCTGGCCCT	TGAAAATCCG	16260
GGGGAGAGGG	TGTAAATCTC	GCGCCGGGCC	GTACCCATAT	CCGCAGCAGG	TCTCCAAGGT	16320
GAACAGCCTC	TGGCATGTTG	GAACAATG	GGTAAGGGAA	GTCGCAAGC	GGGATCCGTA	16380
ACTTCGGGAT	AAGGATTGGC	TCTAAGGGCT	GGGTGGTCCG	GGCTGGGGCG	CGAAGCGGGG	16440
CTGGGCGCGC	GCCCGGGCTG	GACGAGGC	CGCCGCCCTC	TCCCACGTC	GGGGAGACCC	16500
CCCGCTCTT	CCGGCCGGGCC	CCGGCCCTCC	CTCTTCCCCG	CGGGGCCCG	TGTCCCCCCG	16560
CGTCGTCGCC	ACCTCTCTTC	CCCCCTCTT	CTTCCCGTCG	GGGGCGGGGT	GGGGGGTCGG	16620
CGCGCGGGCG	GGGCTCCGGG	CGGGCGGGTC	CAACCCCGCG	GGGGTCCGG	AGCGGGAGGA	16680
ACCAGCGGTC	CCCCGTGGGG	CGGGGGGCC	GGACACTCGG	GGGGCCGGCG	GCGGCGGCCGA	16740
CTCTGGACGC	GAGCCGGGCC	CTTCCCGTGG	ATCGCCTCAG	CTGCGGCGGG	CGTCGCGGCC	16800
GCTCCCGGGG	AGCCCGGGCG	GTGCCGGCGC	GGGTCCCCCTC	CCCAGGGGGC	CTCGCTCCAC	16860
CCCCCCATCG	CCTCTCCCGA	GGTGCCTGGC	GGGGGCGGGC	GGGCGTGTCC	CGCGCGTGTG	16920
GGGGGAACCT	CCGCGTCGGT	GTTCCCCCGC	CGGGTCCGCC	CCCCGGGCCG	CGGTTTCCG	16980
CGCGCGGCC	CCGCGCTCGGC	CGGCGCTAG	CAGCGACTT	AGAACTGGTG	CGGACCAAGGG	17040
GAATCCGACT	GTTTAATTAA	AACAAAGCAT	CGCGAAGGCC	CGCGCGGGGT	GTTGACCGGA	17100

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TGTGATTTCT	GCCCAGTGCT	CTGAATGTCA	AAGTGAAGAA	ATTCAATGAA	GCGCGGGTAA	17160
ACGGCGGGAG	TAACTATGAC	TCTCTTAAGG	TAGCCAAATG	CCTCGTCATC	TAATTAGTGA	17220
CGCGCATGAA	TGGATGAACG	AGATTCCCAC	TGTCCCTACC	TACTATCCAG	CGAAACCACA	17280
GCCAAGGGAA	CGGGCTTGGC	GGAATCAGCG	GGGAAAGAAG	ACCCCTGTTGA	GCTTGACTCT	17340
AGTCTGGCAC	GGTGAAGAGA	CATGAGAGGT	GTAGAATAAG	TGGGAGGCC	CCGGCGCCCG	17400
GCCCCGTCT	CGCGTCGGGG	TCGGGGCACG	CCGGCCTCGC	GGGCCGCCG	TGAAATACCA	17460
CTACTCTCAT	CGTTTTTCA	CTGACCCGGT	GAGGCGGGG	GGCGAGCCC	GAGGGGCTCT	17520
CGCTTCTGGC	GCCAAGCGTC	CGTCCCGCGC	GTGCGGGCGG	GCGCAGACCG	CTCCGGGAC	17580
AGTGCCAGGT	GGGGAGTTTG	ACTGGGGCGG	TACACCTGTC	AAACGGTAAC	GCAGGTGTCC	17640
TAAGGCGAGC	TCAGGGAGGA	CAGAAACCTC	CCGTGGAGCA	GAAGGGCAA	AGCTCGCTTG	17700
ATCTTGATTT	TCAGTACGAA	TACAGACCGT	GAAAGCGGGG	CCTCACGATC	CTTCTGACCT	17760
TTTGGGTTTT	AAGCAGGAGG	TGTCAGAAAA	GTTACACAG	GGATAACTGG	CTTGTGGCGG	17820
CCAAGCGTC	ATAGCGACGT	CGCTTTTGA	TCCTTCGATG	TCGGCTCTTC	CTATCATTGT	17880
GAAGCAGAAT	TCACCAAGCG	TTGGATTGTT	CACCCACTAA	TAGGGAACGT	GAGCTGGGTT	17940
TAGACCGTCG	TGAGACAGGT	TAGTTTACG	CTACTGATGA	TGTGTTGTTG	CCATGGTAAT	18000
CCTGCTCAGT	ACGAGAGGAA	CCGCAAGGTT	AGACATTGG	TGTATGTTG	TGGCTGAGGA	18060
GCCAATGGGG	CGAAGCTACC	ATCTGTGGGA	TTATGACTGA	ACCGCTCTAA	GTCAGAAATCC	18120
GCCCAAGCGG	AACGATACGG	CAGCGCCGAA	GGAGCCTCGG	TTGGCCCCGG	ATAGCCGGGT	18180
CCCCGTCCGT	CCCCTCGGC	GGGGTCCCCG	CGTCGCCCCG	CGGCGGCGCG	GGGTCTCCCC	18240
CCGCCGGGCG	TGGGGACCGG	GGTCCGGTGC	GGAGAGCCGT	TGCTCTGGG	AAACGGGGTG	18300
CGGCCGGAAA	GGGGGCCGCG	CTCTCGCCCCG	TCACGTTGAA	CGCACGTTCG	TGTGGAACCT	18360
GGCGCTAAC	CATTCGTTAGA	CGACCTGCTT	CTGGGTCGGG	GTTCTGTA	TAGCAGAGCA	18420
GCTCCCTCGC	TGCGATCTAT	TGAAAGTCAG	CCCTCGACAC	AAGGGTTTGT	CTCTGCGGGC	18480
TTTCCCGTCG	CACGCCCGCT	CGCTCGCACG	CGACCGTGT	GCCGCCCGGG	CGTCACGGGG	18540
GCGGTCGCC	CGGGCCCCCG	CGGTTGTC	GAACGACCGT	TGTTGTTG	GGGGGGGGAT	18600
CGTCTTCTCC	TCGGTCTCCC	GAGGACGGGT	CGTTTCTCTT	TCCCCCTTC	TGGCTCTCCT	18660
TGGGTGTGGG	AGCCTCGTGC	CGTCGCGACC	GCGGCTTGCC	GTGCGCTG	GCCGCAAGCCC	18720
CTTGCCTCC	GGCCTTGGCC	AAGCCGGAGG	GCGGAGGAGG	GGGATCGGCG	CGGGCGGCGA	18780
CCGCGGGCG	GTGACGACG	GTGGGATCCC	CATCCTCGGC	GCGTCCGTG	GGGACGGCCG	18840
GTTGGAGGGG	CGGGAGGGGT	TTTCCCGTG	AACGCGCGT	TCGGCGCCAG	GCCCTCTGGCG	18900
GCCGGGGGGG	CGCTCTCTCC	GCCCGAGCAT	CCCCACTCCC	GCCCTCTC	TTCGCGCGCC	18960
CGGGCGGGGA	CGTGCAGTAC	AGGGGAGGAT	GTGCGGGTGT	GGAGGCGGAG	AGGGTCCGGC	19020
GCGGCGCCTC	TTCCATTTT	TCCCCCCCCA	CTTCGGAGGT	CGACCAAGTAC	TCCGGGCGAC	19080
ACTTTGTTT	TTTTTTTCC	CCCGATGCTG	GAGGTGCA	AGATGTCGGA	AAGTGTCCCC	19140
CCCCCCCCCC	CCCCCGGCG	CGGAGCGCG	GGGCCACTCT	GGACTCTTTT	TTTTTTTTTT	19200
TTTTTTTTT	TTAAATTCT	GGAACCTTTA	GTCGACCA	TTGTCGCT	TTTACTCCTT	19260
CATATAGGTC	GACCAAGTAC	CCGGGTGGTA	CTTGTCTTT	TTCTGAAAAT	CCCAGAGGTC	19320
GACCAGATAT	CCGAAAGTCC	TCTCTTCCC	TTTACTCTTC	CCCACAGCGA	TTCTCTTTT	19380
TTTTTTTTT	TTTGGTGTGC	CTCTTTTGA	CTTATATACA	TGTAATAGT	GTGTACCTT	19440
ATATACTTAT	AGGAGGAGGT	CGACCAGTAC	TCCGGGCGAC	ACTTTGTTT	TTTTTTTTTT	19500
TCCACCGATG	ATGGAGGTCG	ACCAGATGTC	CGAAAGTGT	CCGTCCCCCCC	CCTCCCCCCC	19560
CCGCGACGCG	GCGGGCTCAC	TCTGGACTCT	TTTTTTTTT	TTTTTTTTT	TTTAAATTTC	19620
TGGAACCTTA	AGGTGACCA	GTTGTCGTC	TTTCACTCAT	TCATATAGGT	CGACCGGTGG	19680
TACTTTGTCT	TTTCTGAAA	ATCGCAGAGG	TCGACAGAT	GTCAGAAAGT	CTGGTGGTGC	19740
ATAAAATTATC	TGATCTAGAT	TTGTTTTCT	TTTTCAGT	TTTGTGTTGT	TTTGTGTTGT	19800
TTTGTGTTGT	TTTGTGTTGT	TTTGTGTTGT	TTTGTGTTGT	TTTGTGTTGT	TTTGTGTTGT	19860
TTTGTGTTGT	GTTGTGTTGT	GTTGTGTTGG	GTTGGGTTGG	GTTGGGTTGG	GTTGGGTTGG	19920
GTTGGGTTGG	GTTGGGTTGT	GTTGGTTGG	TTTGTGTTGT	TTGGTGTG	TGGTTTTGTT	19980
TTGTTGTCG	TTGTTTGTG	TTTGTGTTGG	CGAACAGTTG	TCCCTAACCG	AGTTTTTTG	20040
TACACAAACA	TGCACTTTT	TTAAATTTAA	TTTTTAAAT	AAATGCGAAA	ATCGACCAAT	20100
TATCCCTTC	CTTCTCTCTC	TTTTTAAAT	TTTTTCTTG	TGTGTGTTG	TGTGTGTTG	20160
TGTGTGTTG	TGCGTGTG	TGTGTGTTG	CGTGCAGCGT	GCGCGCGCTC	GTTTTATAAA	20220
TACTTATAAT	AATAGGTCGC	CGGGTGGTGG	TAGCTTCCC	GACTCCAGAG	GCAGAGGCAG	20280
GCAGACTTCT	GAGTTGAGG	CCAGCCTGGT	CTACAGAGGA	ACCCCTGTC	AAAAAATGAA	20340
AATAAAATACA	TACATACATA	CATACATACA	TACATACATA	CATACATACA	TACATATGAG	20400
GTTGACCAGT	TGTCAATCT	TTAGAATTT	TTTTTTAATT	AATGTGATAG	AGAGATAGAT	20460
AATAGATAGA	TGGATAGAGT	GATACAAATA	TAGGTTTTT	TTTCAGTAA	TATGAGGTTG	20520
ATTAACCACT	TTTCCCTTTT	TTAGGTTTTT	TTTTTTTCCC	CTGTCATGT	GGTTGCTGGG	20580
ATTTGAACTC	AGGACCCCTGG	CAGGTCAACT	GGAAAACGTG	TTTCTATAT	ATATAAATAG	20640
TGGTCTGTC	GCTGTTGTT	TGTTTGTG	CTTGCTTGCT	TGCTTGCTTG	CTTGCTTGCT	20700
TCCTTTCTGA	GACAGTATT	CTCTGTGAA	CCTGGTGGCCC	TGAAACTCAC	20760	

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TCTGTAGACC	AGCCTGGCCT	CAATCGAACT	CAGAAATCCT	CCTGCCTCTT	GTCTACCTCC	20820
CAATTGGA	GTAAAGGTGT	GCTACACCAC	TGCCCTGGCAT	TATTATCATT	ATCATTATTA	20880
ATTTTATTAT	TAGACAGAAC	GAAATCAACT	AGTTGGTCCT	GTTCGTTAA	TTCATTTGAA	20940
ATTAGTTGGA	CCAATTAGTT	GGCTGGTTTG	GGAGGTTCT	TTTGTTCG	ATTTGGGTGT	21000
TTGTGGGGCT	GGGGATCAGG	TATCTCAACG	GAATGCATGA	AGGTTAAGGT	GAGATGGCTC	21060
GATTTTGT	AAGATTACTT	TTCTTAGTCT	GAGGAAAAAA	AAAATAATA	TTGGGCTACG	21120
TTTCATTGCT	TCATTCTAT	TTCTCTTCT	TTCTTCTT	CTTTCAGATA	AGGAGGTCGG	21180
CCAGTTCCCTC	CTGCCTCTG	GAAGATGTAG	GCATTGCATT	GGGAAAAGCA	TTGTTTGAGA	21240
GATGTGCTAG	TGAACCAGAG	AGTTTGATG	TCAAGCCGTA	TAATGTTTAT	TACAATATAG	21300
AAAAGTTCTA	ACAAAGTGT	CTTTAACTT	TTTTTTTTT	TTTCTCCTTC	TACTTCTACT	21360
TGTTCTCACT	CTGCCACCAA	CGCGCTTGT	ACATTGAATG	TGAGCTTGT	TTTGCTTAAC	21420
AGACATATAT	TTTTCTTTT	GGTTTTGCTT	GACATGGTT	CCCTTTCTAT	CCGTGCAGGG	21480
TTCCCAGACG	GCCTTTGAG	AATAAAATGG	GAGGCCAGAA	CCAAAGTCTT	TTGAATAAAG	21540
CACCAACACT	CTAACCTGTT	TGGCTTTT	CCTTCCCAAG	GCACAGATCT	TTCCCAGCAT	21600
GGAAAAGCAT	GTAGCAGTTG	TAGGACACAC	TAGACGAGAG	CACCAAGATCT	CATTGTGGGT	21660
GGTTGTGAAC	CACCCACCAT	GTGGTTGCCT	GGGATTGAA	CTCAGGATCT	TCAGAAGACG	21720
AGTCAGGGCT	CTAAACCGAT	GAGCCATCTC	TCCAGCCCTC	CTACATTCT	TCTTAAGGCA	21780
TGAATGATCC	CAGCATGGGA	AGACAGTCTG	CCCTCTTGT	GGTATATCAC	CATATACTCA	21840
ATAAAATAAT	GAAATGAATG	AAAGTCTCCAC	GTATTTATT	CTTCGAGCTA	TCTAAATTCT	21900
CTCACAGCAC	CTCCCCCTCC	CCCACACTG	CTTCTCCCT	ATGTTGGGT	GGGGCTGGGG	21960
GAGGGGTGG	GTGGGGCAG	GGATCTGCAT	GTCTTCTTGC	AGGTCTGTGA	ACTATTGCG	22020
ATGGCCTGGT	TCTCTGAACT	GTTGAGCCCT	GTCTATCCAG	AGGCTGACTG	GCTAGTTTC	22080
TACCTGAAGT	CCCTGAGTGA	TGATTTCCCT	GTGAATT			22118

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42999 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTGACACGC	TGTCTCTGG	CGACCTGTCG	TCGGAGAGGT	TGGGCCTCCG	GATGCGCGCG	60
GGGCTCTGGC	CTCACGGTGA	CCGGCTAGCC	GGCCGCGCTC	CTGCCTTGAG	CCGCTGCG	120
CGGCCCGCGG	GCCTGCTGTT	CTCTCGCGCG	TCCGAGCGTC	CCGACTCCCG	GTGCCGGCCC	180
GGGTCCGGGT	CTCTGACCCA	CCCAGGGGCG	GGGGGAAAGG	CGGCGAGGGC	CACCGTGC	240
CGTGCCTCT	CCGCTGCGGG	CGCCCCGGGC	GGCGCACAAAC	CCCACCCGCT	GGCTCCGTGC	300
CGTGCCTGTC	AGGCGTTCTC	GTCTCCCGGG	GGTTGTCGGC	CGCCCCCTTC	CCGGAGTGGG	360
GGGTGGCCGG	AGCCGATCGG	CTCGCTGGCC	GGCCGGCCCTC	CGCTCCCGGG	GGGCTCTTCG	420
ATCGATGTGG	TGACGTCGTG	CTCTCCCGGG	CGGGTCCCGA	GGCCGCGACGG	GCGAGGGGCG	480
GACGTTCGTG	GGCAACGGGA	CGCTCTTCT	CGCTCCGGCC	GGCGGGTCCC	CTCGTCTGCT	540
CCTCTCCCG	CCCGCCGGCC	GGCGTGTGGG	AAGGCGTGGG	GTGCGGACCC	CGGCCCGACC	600
TCGCCGTC	GGCCGCCGCC	TTCGCTTCG	GGGTGCGGGC	CGCGGGGGTC	CTCTGACCGC	660
GCAGACAGCC	CTGCCTGTCG	CCTCCAGTGG	TTGTCGACTT	GGGGCGGCC	CCCCTCCGCG	720
GCGGTGGGG	TGCCGTCCCG	CCGGCCCGTC	GTGCTGCCCT	CTCGGGGGGG	GTGGCGCGA	780
GCGTCGGCTC	CGCCTGGGCC	CTTGCCTGTC	TCCTGGAGCG	CTCCGGGTTG	TCCCTCAGGT	840
GCCCGAGGCC	GAACGGTGGT	GTGTCGTTCC	CGCCCCCGGC	GCCCCCTCCT	CCGGTCGCCG	900
CCGCGGTGTC	CGCCGCTGGG	TCCTGAGGGA	GCTCGTCGGT	GTGGGGTTCG	AGGCGGTTTG	960
AGTGAGACGA	GACGAGACGC	GCCCCCTCCCA	CGCGGGGAAG	GGCGCCCGCC	TGCTCTCGGT	1020
GAGCGCACGT	CCCGTGTCTCC	CCTCTGGCGG	GTGCGCGCGG	GCCGTGTGAG	CGATCGCGGT	1080
GGGTTCGGGC	CGGTGTGACG	CGTGCCTGGG	CGGGCCGCCG	AGGGGCTGCC	GTTCTGCCTC	1140
CGACCGGTG	TGTGTGGGTT	GACTTCGGAG	GCGCTCTGCC	TCGGAAGGAA	GGAGGTGGGT	1200
GGACGGGGGG	GCCTGGTGGG	GTTGCGCGCA	CGCGCGCACC	GGCGGGGCC	CCGCCCCGAA	1260

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CGCGAACGCT	CGAGGGTGGCC	GCGCGCAGGT	GTTTCCTCGT	ACCGCAGGGC	CCCCTCCCTT	1320
CCCCAGGCGT	CCCTCGGCCG	CTCTGCAGGC	CCGAGGAGGA	GCGGCTGGCG	GGTGGGGGGA	1380
GTGTGACCCA	CCCTCGGTGA	AAAAAGCTT	CTCTAGCGAT	CTGAGAGGCG	TGCCTGGGG	1440
GTACCGGATC	CCCCGGGCGG	CCGCCTCTGT	CTCTGCCTCC	GTTATGGTAG	CGCTGCCGTA	1500
GCGACCCGCT	CGCAAGAGGAC	CCTCCTCCGC	TTCCCCCTCG	ACGGGGTTGG	GGGGGAGAAG	1560
CGAGGGTTCC	GCCGGCCACC	GCGGTGGTGG	CCGAGTGCAG	CTCGTCGCCT	ACTGTGGCCC	1620
GCGCCTCCCC	CTTCGAGTC	GGGGGAGGAT	CCCGCGGGGC	CGGGCCCCGGC	GCTCCCACCC	1680
AGCGGGTTGG	GACCGGGCGG	CCGGCGGGCG	GTGGGTGTGC	GCGCCCGGGCG	CTCTGTCCGG	1740
CGCGTGACCC	CCTCCGTCCG	CGAGTCGGCT	CTCCGCCCGC	TCCCCTGCCG	AGTCGTGACC	1800
GGTGCAGACG	ACCGCGTTTG	CGTGGCACGG	GGTCGGGCC	GCCTGGCCCT	GGGAAAGCGT	1860
CCCACGGTGG	GGGCGCGCCG	GTCTCCCGGA	GCAGGGACCGG	GTCGGAGGAT	GGACGAGAAAT	1920
CACGAGCGAC	GGTGGTGGTG	CGGTGTCGGG	TTCGTGGCTG	CGGTCGCTCC	GGGGCCCCCG	1980
GTGGCGGGGC	CCCAGGGCTC	GCGAGGCGGT	TCTCGGGGGG	GGCCGAGGGC	CGTCCGGCGT	2040
CCCAGGCGGG	GCGCCGCGGG	ACCGCCCTCG	TGTCTGTGGC	GGTGGGATCC	CGCGGCCGTG	2100
TTTTCTGTT	GGCCCGGCCG	TGCCTGAGGT	TTCTCCCCGA	GCGCCGCGCT	CTGCGGGCTC	2160
CCGGGTGCC	TTGCGCTCGC	GGTCCCCCGC	CCTCGCCCGT	CTGTGCCCCC	TTCCCCGCC	2220
GGCCCGCTGG	GATGCTCTTC	TTCCCCCGA	GCGGCTCAC	GGCTTCACGT	CGGTTGGTGG	2280
CCCCCGCTGG	GACCGAACCC	GCGACCGCCT	CGTGGGGCGC	CGCCGCCGCC	CACTGATCGG	2340
CCCGCGCTCC	GCGTCCCCCG	GCGCGCGCT	TGGGGACCGG	GTCGGTGGCG	CGCCGCGTGG	2400
GGCCCGGTGG	GCTTCCCGGA	GGGTTCCGGG	GGTCGGCCTG	CGGCGCGTGC	GGGGGAGGAG	2460
ACGGTTCCGG	GGGACCGGGC	GCGGCTCGGG	CGGGCGCGGT	GGTGGGGGGA	GCCGCGGGGA	2520
TCGCCGAGGG	CCGGTCGGCC	GCCCCGGGTG	CCCCGCGGTG	CCGCCGGCGG	CGGTGAGGCC	2580
CCGCGCGTGT	GTCCCCGCTG	CGGTCGGCCG	CGCTCGAGGG	GTCCCCGTGG	CGTCCTCCCTC	2640
CCCGCCGGCC	GCCTTTCTCG	CGCCTTCCCC	GTCGCCCCGG	CCTCGCCCGT	GGTCTCTCGT	2700
CTTCTCCCGG	CCCGCTCTTC	CGAACCGGGT	CGGCGCGTCC	CCCGGGTGCG	CCTCGCTTCC	2760
CGGGGCTGCC	GGGGCCCCCTC	CCCGAGGCGT	CGCTCCCGGG	CGTCGGCGTC	GGGGAGAGCC	2820
CGTCCTCTCC	GGCGTGGCGTC	GCCCCGTTG	GCGCGCGCGT	CGCCCGCAGC	CGGGCCCGGT	2880
GGTCCTCTCC	GGACAGGCGT	TCGTGCGACG	TGTGGCTGG	GTCGACCTCC	GCCTTGCCTGG	2940
TCGCTCGCCC	TCTCCCCGGG	TCGGGGGGTG	GGGCCCCGGG	CGGGGCGCTG	GCCCCCGGTG	3000
CTGCCTCCCG	TCCCCGGCGG	GGGCGGGCGC	GCCGGCCGCC	CTCGGTCGCG	CTCCCTTGGC	3060
CGTCGTGTGG	CGTGTGCCAC	CCCTGCGCCG	GCGCCCGCCG	CGGGGGCTCG	GAGCCGGGCT	3120
TCGGCCGGGC	CCCAGGGCCCT	CGACCGGACC	GGCTGCGCGG	GCGCTGCGGC	CGCACGGCGC	3180
GAATGTCCCC	GGGCGGGGCA	CCGCGGTCCG	CCTCTCGCTC	GCGCCCGCGA	CGTCGGGGCC	3240
GCCCCCGCGG	GGGGCGGGAG	CGCCGTCCCC	GCTCTGCCGC	CGCCCCCGGG	CGCCGGCCGC	3300
GGCGCGCGC	GGGTGGCCGC	CGGTCCCTCC	CGGCCGCCGG	CGCGGGGTGCG	GGCCGTCCGC	3360
CTCTCTCGGG	GGGGCGCGA	CGAACAGGCG	TGCGGGTCT	GTGGCGCGGG	GCCCCCGGTG	3420
GTCTGTGCG	GTGGGGGGCG	GGTGGTTGGG	GGCTCCGGGT	CGCCCGCGCC	GGGGCCGGCC	3480
CCACCGGTCC	GGGCCGCCGC	CCCCCGCGCC	GCTCGTCTCC	TCCCCCTCCG	CGGTCCCGGG	3540
CCCGTCCGTC	CGTCCGTCGG	TCGTCTCTC	CGCTTGCGGG	GCGCCGGGGC	CGTCTCTCGG	3600
AGGCCCCCG	GCGGCCGTC	CGGCCCGCGC	GGGGGCTCGC	CGCGCTCTAC	CTTACCTACC	3660
TGGTTGATCC	TGCCAGTAGC	ATATGTTGT	CTCAAAGATT	AAGCCATGCA	TGTCTAAGTA	3720
CGCACGGCCG	GTACAGTGAA	ACTGCGAATG	GCTCATTAAA	TCAGTTATGG	TTCTTTGGT	3780
CGCTCGCTCC	TCTCTTACTT	GGATAACTGT	GGTAATTCTA	GAGCTAATAC	ATGCCGACGG	3840
GGCTGTACCC	CCTTCGCGGG	GGGGATGCGT	GCATTTATCA	GATCAAACCC	AACCCGGTCA	3900
GCCCCCTCTC	GGCCCCGGCC	GGGGGGCGGG	CGCCGGCGGC	TTTGGTACT	CTAGATAACC	3960
TCGGGCGAT	CGCACGCCCG	CGTGGCGGCC	GACGACCCAT	TGAAACGTCT	GCCCTATCAA	4020
CTTCGATGG	TAGTCGCCGT	GCCTTACCATG	GTGACCACGG	GTGACGGGGA	ATCAGGGTTC	4080
GATTCCGGAG	AGGGAGCGT	AGAAACGGCT	ACCACATCCA	AGGAAGGCAG	CAGCGCGCCA	4140
AATTACCCAC	TCCCCACCCG	GGGAGGTAGT	GACGAAAAT	AACAATACAG	GACTCTTCG	4200
AGGCCCTGTA	ATTGGAATGA	GTCCACTTTA	AATCCTTAA	CGAGGATCCA	TTGGAGGGCA	4260
AGTCTGGTC	CAGCAGCCGC	GGTAATTCCA	GCTCCAATAG	CGTATATTAA	AGTTGCTGCA	4320
GTAAAAAGC	TCGTAGTTGG	ATCTTGGGAG	CGGGCGGGCG	GTCCGCCGCG	AGGCAGGCCA	4380
CCGCCCCGTCC	CCGCCCCCTG	CCTCTCGCG	CCCCCTCGAT	GCTCTTAGCT	GAGTGTCCCG	4440
CGGGGCGCGA	AGCGTTTACT	TTGAAAAAAAT	TAGAGTGTTC	AAAGCAGGCC	CGAGCCGCC	4500
GGATAACCGCA	GCTAGGAATA	ATGGAATAGG	ACCGCGGTTG	TATTTGTTG	GTGGCGGAA	4560
CTGAGGGCCAT	GATTAAGAGG	GACGGCCGGG	GGCATTCTGTA	TTGCGCCGCT	AGAGGTGAAA	4620
TTCTTGGACC	GGCGCAAGAC	GGACCGAGGC	GAAAGCATTT	GCCAAGAATG	TTTTCATTA	4680
TCAAGAACGA	AAAGTCGGAGG	TTCGAAGACG	ATCAGATACC	GTCGTAGTTC	CGACCATAAA	4740
CGATGCGGAC	CGGCGATGCG	GGGGCGTTAT	TCCCAGTGACC	CGCCGGGCAG	CTTCCGGGAA	4800
ACCAAAGTCT	TTGGGGTCCG	GGGGGAGTAT	GGTTGCAAAG	CTGAAACTTA	AAGGAATTGA	4860
CGGAAGGGCA	CCACCAAGGAG	TGGAGCCTGC	GGCTTAATT	GACTCAACAC	GGGAAACCTC	4920

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ACCCGGCCCC	GACACGGACA	GGATTGACAG	ATTGATAGCT	CTTTCTCGAT	TCCGTGGGTG	4980
GTGGTGCATG	GCCGTTCTTA	GTGGTGGAG	CGATTGTC	GGTTAATTCC	GATAACGAAC	5040
GAGACTCTGG	CATGCTAACT	AGTTACGCGA	CCCCCGAGCG	GTCGGCGTCC	CCCAACTTCT	5100
TAGAGGGACA	AGTGGCGTTC	AGCCACCGA	GATTGAGCAA	TAACAGGTCT	GTGATGCCCT	5160
TAGATGTCCG	GGGCTGCACG	CGCGCTACAC	TGACTGGCTC	AGCGTGTGCC	TACCCCTACGC	5220
CGGCAGGCGC	GGGTAACCCG	TTGAACCCCA	TTCGTGATGG	GGATCGGGGA	TTGCAATTAT	5280
TCCCCATGAA	CGAGGGATT	CCCGAGTAAG	TGCGGGTCAT	AAGCTTGCCT	TGATTAAGTC	5340
CCTGCCCTT	GTACACACCG	CCCCTCGCTA	CTACCGATTG	GATGGTTTAG	TGAGGCCCTC	5400
GGATCGGCC	CGCCGGGTC	GGCCCACCGC	CCTGGCGGAG	CGCTGAGAAG	ACGGTCGAAC	5460
TTGACTATCT	AGAGGAAGTA	AAAGTCGTA	CAAGGTTTCC	GTAGGTGAAC	CTGCGGAAGG	5520
ATCATTAACG	GAGCCCGGAG	GGCAGGGCC	GCGCGGGCGC	CGCCGCCGCC	CGCGCTTCC	5580
CTCCGCACAC	CCACCCCCCC	ACCGCGACGC	GCGCGTGC	CGGGCGGGGC	CGCGTGC	5640
GTTCGTTCG	TGCGCTCGTC	GTTCGGCC	CGGGCGGCC	GCGCGAGAG	CCGAGAAC	5700
GGGAGGGAGA	CGGGGGGAG	AGAGAGAGAG	AGAGAGAGAG	AGAGAGAGAG	AGAGAGAGAA	5760
AGAAGGGCGT	GTGCGTTGGTG	TGCGCGTGT	GTGGGGCCGG	CGGGCGGC	GGAGCGGTCC	5820
CCGGCCGCGG	CCCCGACGAC	GTGGGTGTC	CGGGCGCGG	GGCGGTTCT	CGGCGGCGTC	5880
GCGGCGGGTC	TGGGGGGGTC	TCGGTGCCT	CTCCCCGCG	GGGGCCCCTC	GTCCGGCCCC	5940
GCCGCGCCGG	CTCCCCGTCT	TCGGGGCCGG	CCGGATTCCC	GTGCGCTCCG	CCGCGCCGCT	6000
CCGCGCCGCC	GGCACCGGCC	CCGCTCGCTC	TCCCCGGCCT	TCCCCTCGTAG	CGCTCTCGAG	6060
GGTCGGGGGC	CGGACGCCGG	TCCCCCTCCC	CGCCTCCCTCG	TCCGCCCCCCC	CGCCGTC	6120
GTACCTAGCG	CGTTCCGGCG	CGGAGGTTTA	AAGACCCCTT	GGGGGGATCG	CCCCTCCGCC	6180
CGTGGGTCCG	GGGGCGGTGGT	GGGGCGCCGG	GGGAGTCCC	TGGGGAGGGG	CCCCGCCCT	6240
CCCGCGCCCT	CACCGCGGAC	TCCGCTCCC	GGCCGGGGCC	GCGCCGCCGC	CGCCGCCGCG	6300
GCGGCCGTCG	GGTGGGGCT	TTACCGGCG	GCGTCGCGC	GCCTGCGCG	CGTGTGGCGT	6360
GCGCCCCGCG	CCGTGGGGGC	GGGAACCCCC	GGGCGCCTGT	GGGGTGGTGT	CCGCGCTCGC	6420
CCCCCGCTGG	GCGGCGCGCG	CCTCCCCGTG	GTGTGAAACC	TTCCGACCCC	TCTCCGGAGT	6480
CGGGTCCCGT	TTGCTGTCTC	GTCTGGCCGG	CCTGAGGCAA	CCCCCTCTCC	TCTTGGGCGG	6540
GGGGGGCGGG	GGGACGTGCC	GCGCCAGGAA	GGGCTCCCTC	CCGGTGC	GTGCGGAGCG	6600
CCCTCGCCAA	ATCGACCTCG	TACGACTCTT	AGCGGTGGAT	CACTCGGCTC	GTGCGTCGAT	6660
GAAGAACGCA	GCTAGCTGCG	AGAATTAAATG	TGAATTGCA	GACACATTGA	TCATCGACAC	6720
TTCGAACGCA	CTTGCGGCCC	CGGGTTCTC	CGGGGGCTAC	GCCTGCTCTGA	CGCTCGCTTG	6780
CCGATCAATC	GCCCCGGGGG	TGCGCTCCGG	CTCTCGGGG	TGCGCGCTG	GGGGTTCCCT	6840
CGCAGGGCCC	GCGGGGGGCC	CTCCGTCCTC	CTAACCGCAG	ACCCGGCGGC	GTCCGCCCTC	6900
CTCTTGCCTC	CGCGCCCGCC	CCTTCCCCCT	CCCCCGCGG	GCCTGCGTG	GTACCGCGTC	6960
GGGTGGCGGG	GGGGAGAGGG	GGGCGCGCCC	GGCTGAGAGA	GACGGGGAGG	GCGCGCCGCG	7020
CGCCGGAAGA	CGGAGAGGG	AAGAGAGAGC	CGGCTGGGC	CGAGTTCCC	TGGCCGCCGC	7080
CTGCGGTCCG	GGTTCTCTCC	TCGGGGGCT	CCCTCGCGCC	GCGCGCGGCT	CGGGGTTCGG	7140
GGTTCGTCGG	CCCCGGCCGG	GTGGAAGGTC	CCGTGCCCCT	CGTCGTCGTC	GTGCGCGTC	7200
GTCGGCGGTG	GGGGCGTGT	GCGTGCCTG	TGGTGGTGGG	GGAGGAGGAA	GGCGGGTCCG	7260
GAAGGGGAAG	GGTGCCGGCG	GGGAGAGAGG	GTGGGGGAG	CGCGTCCC	TGCGGGCGGT	7320
TCCGCCGCC	GCCCCCGGTG	GCGGCCGGC	GTCCCGCGA	CGGGCGCGCT	CCCGCGCCCC	7380
TCCTCCTCCC	CGCCGCCCT	CCTCGAGGC	CCCGCCGTC	CTCCTCGCC	TCCCCCGCG	7440
TACGCGCGCG	CGCCCCCGCC	CCCCTCGC	CTCGCGCGC	GTCGGCCGGG	GCGGGGAGCC	7500
CGCCCCCGCG	CCCCCGCTG	GCGCGCGC	CGGGGTTCGC	GTGTCCTCCGG	CGCGCACCCG	7560
CGGGACGCG	CGGTGTCGTC	CGCCGTCGCG	CGCCCGCCTC	CGGCTCGCG	CGCGGCCGCG	7620
CCGCGCCGGG	GCCCCGTCCC	GAGCTTCCG	GTCGGGGCGG	CGCGCTCC	CGGCCGCGTC	7680
CTCGGACCCG	TCCCCCGAC	CTCCGCGGG	GAGACCGC	GGGGCGTGCG	GCGCCCGTCC	7740
CGCCCCCGGC	CCGTGCCCT	CCCTCCGTC	GTCCTCGCTC	GGCGGGGCGG	CGCGGGGGCG	7800
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GTCCCCCTCC	GAGACCGC	CTCAGATCG	ACGTGGCGAC	CCGCTGAATT	TAAGCATATT	7980
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GAGCCCCAGG	CGGAATCCCC	GCCCCCGGG	GGCGGGGACA	TGTGGCGTAC	GGAAGACCCG	8100
CTCCCCGGCG	CCGCTCGTGG	GGGGCCAAG	TCTTCTGAT	CGAGGCCCAG	CCCGTGGACG	8160
GTGTGAGGCC	GGTAGCGGCC	GGCGCGCGC	CGGGTCTTC	CGGAGTC	TTGCTGGGA	8220
ATGCAGCCA	AAGGGGTGG	TAAACTCCAT	CTAACCGCTAA	ATACCGGCAC	GAGACCGATA	8280
GTCAACAAAGT	ACCGTAAGGG	AAAGTTGAAA	AGAACCTTG	AGAGAGAGTT	CAAGAGGGCG	8340
TGAAACC GTT	AAGAGGTAAA	CGGGTGGGGT	CGCGCAGTC	CGCCCGGAGG	ATTCAACCCG	8400
GCGGC GGCGT	CGGCCGTGTC	GGCGGCCGG	CGGATCTTC	CCGCCCCCG	TTCCCTCCGA	8460
CCCCTCCACC	CGCCCTCCCT	TCCCCCGCG	CCCCTCCCTC	TCCTCCCCGG	AGGGGGCGGG	8520
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CCCGACCGGC	GACCGGCCGC	CGCCGGGCGC	ATTTCCACCG	CGGC GG TGCG	CCGCGACC GG	8640
CTCCGGGACG	GCTGGGAAGG	CCC GG CGGGG	AAGGTGGCTC	GGGGGGGCC	GTCCGTC	8700
CCGTCTCCT	CCTCCCCCGT	CTCCGCC	CGGCC	GGCGGCGG	GGAGGGCG	8760
CGGGTGGGG	CGGCGGCGG	GGCGGCGG	GGGGCGGG	CGGGGGCGG	GGGACCGAAA	8820
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AGCGAGACCC	GTCGCCGCGC	TCTCCCCCT	CCC GG CGCC	ACCCCGCGG	GAATCCCC	8940
GCGAGGGGG	TCTCCCCCGC	GGGGCGCGC	GGCGGTCTC	TCGTTGGGG	GCGGGGCCAC	9000
CCCTCCCACG	GCGCGACCG	TCTCCCACCC	CTCCTCCC	CGCCCCCGCC	CCGGCGACGG	9060
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GTCGCGCCGT	CGGGCCC	GGAGGTTCTC	TCG	GGCCGCTCC	CCCGAAGAGG	9180
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TGAAACACGG	ACCAAGGAGT	CTAACACGTG	CGCGAGT	GGGCTCGC	GAAAGCCGCC	9300
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TCCAGTCCGC	CGAGGGCGCA	CCACCGG	GTCTCGCC	CCGCGCCGGG	GAGGTGGAGC	9420
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AGGAAACTCT	GGTGGAGGTC	CGTAGCGG	CTGACGT	AA	ATCGGTC	9540
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GGATAGCTGG	CGCTCTCGCA	GACCCGACG	ACCCCGC	CGCA	TTTCTCA	9660
CGAATGATT	GAGGTCTTGG	GGCGGAAACG	ATCTCAAC	ATTCTCAAC	TTTAAATGGG	9720
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TTTGGTAAG	CAGAACTGGC	GCTGCGG	GAT	GGCTTAAG	GCGCCC	9840
CCGACGCTCA	TCAGACCCC	AAAAAGGTG	TGGT	TGATAT	ACGGTGCCA	9900
TGGAAGTCGG	AATCCGCTAA	GGAGTGTG	ACA	ACTCACC	ACTAGCC	9960
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TCCTCCGCC	CACGCC	CCCC	CGGAG	CCCC	CGGAGCTAC	10200
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CAGGTGAGA	TCTTGGTGGT	AGTAGCAA	ATT	CAAACG	GAAC	10320
GAGAAGGGTT	CCATGTGAAC	AGCAGT	GAT	GGG	TTGAA	10380
AGCGCCGTT	CGAAGGGACG	GGCGATGG	TCC	GG	AGACAGCAGG	10440
CGGGTTCA	TCCCCGAATC	CGGAGTGG	GAGA	TGGG	GGCGAGGCG	10500
TAACCGGACC	GATCCC	AAGCCGG	GAG	CCC	TCTTCTTGT	10560
GAAGGGCAGG	GC	GGCC	GAG	GGG	GGCGAAG	10620
GTCGGGTT	CGGGCGG	ATGGG	GG	CC	GGCC	10680
GTGTAAATCT	CGGCC	CGG	CG	GG	GGAGAG	10740
CTGGCATGTT	GGAA	AC	CTT	GG	GGAG	10800
TAAGGATTGG	CTCTAAGG	AGG	GGG	GGG	GGG	10860
CGCCGCGG	GGACGAGG	GGG	GGG	GGG	GGG	10920
CCCCCGCCC	ACCCGCG	GGCG	GGG	GGG	GGG	10980
CTCTCCCC	CTCCCCG	TCCC	CCC	CCC	CCC	11040
GGGGAGAAGG	GTCGGGCGG	CAGGG	GGG	GGG	GGG	11100
AGGTCCCCG	GAGGGGGC	CCGGG	GGG	GGG	GGG	11160
GCGAGCGGG	CCCTCCC	GGG	GGG	GGG	GGG	11220
GGAGCGCGG	GGCGCG	GGG	GGG	GGG	GGG	11280
GTCGGCTGG	GGCGGAGG	GTC	GGG	GGG	GGG	11340
TCGTCCCC	GCCCTACCC	CCC	GGG	GGG	GGG	11400
GCGCGCGG	GGCGCG	GGC	GGG	GGG	GGG	11460
CGCCCCCGG	GCCCGG	CGC	GGG	GGG	GGG	11520
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CCTCGTCATC	TAATTAGTGA	TAAC	ATG	GGG	GGG	11760
TACTATCCAG	CGAAACCACA	CGCG	CAT	GGG	GGG	11820
ACCCCTGTTGA	GCTTGACTCT	AGT	CTG	GGG	GGG	11880
TGGGAGGGCC	CCGGCGCC	CCC	GGG	GGG	GGG	11940
CTTGC	GGGG	GGG	GGG	GGG	GGG	12000
GGGGGGGCGA	GCCCAGGGGG	TACCA	CTG	GGG	GGG	12060
ACCCGCTCCG	GGGACAGTGC	AGGT	GGG	GGG	GGG	12120
GTAACGCAGG	TGTCCTAAGG	CGAG	CTC	GGG	GGG	12180
GCAAAAGCTC	GCTTGATCTT	GATT	TTG	GGG	GGG	12240

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TGTTGCATG	GTAATCCTGC	TCAGTACGAG	AGGAACCGCA	GGTCAGACA	TTTGGTGTAT	12540
GTGCTTGGCT	GAGGAGCCAA	TGGGGCGAAG	CTACCATCTG	TGGGATTATG	ACTGAACGCC	12600
TCTAAGTCAG	AATCCCAGCC	AGGCGAACGA	TACGGCAGCG	CCCGGGAGCC	TCGGTTGGCC	12660
TCGGATAGCC	GGTCCCCCGC	CTGTCCCCGC	CGCGGGCCG	CCCCCCCCCTC	CACGCGCCCC	12720
GCCGCGGGAG	GGCGCGTGCC	CCGCCGCGCG	CGGGGACCGG	GGTCCGGTGC	GGAGTGCCT	12780
TCGTCTGGG	AAACGGGGCG	CGGCCGGAAA	GGCGGCCGCC	CCCTCGCCCG	TCACGCACCG	12840
CACGTTCTG	GGGAACCTGG	CGCTAAACCA	TTCGTAACGAC	ACCTGCTTCT	GGGTCGGGGT	12900
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TCCCCGTTCA	CGCCGGGGCG	GCTCGTCCGC	TCCGGGCCGG	GACGGGGTCC	GGGGAGCGTG	13200
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CCTCCGGGGT	CGACCGCCCTG	CGCCCGCGGG	CGTGAAGACTC	AGCGGCGTCT	CGCCGTGTCC	13500
CGGGTGCACC	GGGGCCTTCT	CCACCGAGCG	CGGGGTGTAGG	AGTGCCTGTC	GGGACGAACC	13560
GCAACCGGAG	CGGCCCCGTC	TCGGTGGCA	CCTCCGGGGT	CGACCACTGT	CCGCCCGCGA	13620
GCTCCGGACT	TAGCCGGCGT	CTGCACGTGT	CCCGGGTCGA	CCAGCAGGCC	GCGCCCGGAC	13680
GCAGCGCGC	ACGACAGCGA	GGGGTGTGAT	TCCCCCTCGC	GCGCCCGCGC	CTCCACCGGC	13740
CTCGCCCCGC	GGTGGAGCTG	GGACACAGCG	GAACCTCCCTC	TCCCCACATT	TTTCAGCCCC	13800
CACCGCGAGT	TTGCGTCCGC	GGGACCTTAA	AGAGGGAGTC	ACTGCTGCCG	TCAGCCAGTA	13860
CTGCTCTCTC	CTTTTTCGCT	TTTAGGTTTT	GCTTGCTTT	TTTTTTTTTT	TTTTTTTTTT	13920
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CTTCTTGTGT	TCTCTCTCTG	CTCTTCCCTCT	GTCTGTCTCT	CTCTCTCTCT	CTCTCTCTGT	14040
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GTCGCTCTCG	CGCTCTCGCT	CTCTCTCTGT	CTCTGTCTGT	GTCTCTCTCT	CTCCCTCCCT	14220
CCCTCCCTCC	CTCCCTCCCT	CCCTCCCTCT	CCTTGGCGCC	TTCTCGGCTC	TTGAGACTTA	14280
GCCGCTGTCT	CGCCGTACCC	CGGGTCGACC	GGCGGGCGTT	CTCCACCGAG	GGCGTGCACA	14340
CAGTCCCCGT	CGGGACGAGC	CGGACCCGCC	CGTCCCCGT	CTCGGTGCGC	ACCTCCGGGG	14400
TCGACCAGCT	GGCGCCCGCG	AGCTCCGGAC	TTAGCGGCG	TCTGCACGTG	TCCCGGGTCG	14460
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GCGCCCGCGC	CTCCACCGGC	CTGGGCCCGC	CGTGGAGCTG	GGACCAACCGG	GAACTCCCTC	14580
TCCTACATT	TTTCAGGCC	CACCGCGAGT	TTGCGTCCGC	GGGACCTTAA	AGAGGGAGTC	14640
ACTGCTGCCG	TCAGCCAGTA	CTGCTCTCTC	CTTTTTCGCT	TTTAGGTTTT	GCTTGCTTT	14700
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TCTCTGTCTC	TCTCTCTCTG	TCTCTCTCTC	CTCCCTCCCT	CCTTGGTGCC	TTCTCGGCTC	15540
GCTGCTGCTG	CTGCTCTCTG	CTCCACGGTT	CAAGCAAACA	GCAAGTTTC	TATTTCGAGT	15600
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CACACACACA	CACACACACA	CACACACACA	CACACACACA	CACACACACA	GTAGTGTATAA	15840
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AATATGTATC	GAAGCCCCAT	TTCATTTACA	TACACGTGTA	TGTATATCCT	TCCTCCCTTC	15960
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CCGCCTGGTC	TTCTGTCTCT	GCGCTCTGGT	GACCTCAGCC	TCCCCTAATAG	CTGGGACTAC	16080
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CAGCTTACGT	GGGCTCGGGT	GCGGTGGGGT	GGGGTGGGGT	GGGGTGGGGT	GCAGAGAAAA	16200
CGATTGATTG	CGATCTCAAT	TGCCTTTAG	CTTCATTCAAT	ACCCTGTTAT	TTGCTCGTT	16260
ATTCTCATGG	GTTCTCTGT	GTCATTGTC	CGTTCACTCGT	TTGCTTGCT	GCTTGCTGT	16320
TTATTTCCCT	CCTTCCTTC	TTCTCTCTC	CCTCTCTC	TTCTCTCTT	CCCTCCCTTA	16380
CTGGCAGGGT	CTTCCTCTGT	CTCTGCC	CAGGATCACC	CCAACCTCAA	CGCTTTGGAC	16440
CGACCAAACG	GTCGTTCTGC	CTCTGATCCC	TCCCCTACCC	ATTACCTGAG	ACTACAGGCG	16500
CGCACCAACCA	CACCGGCTGA	CTTTTATGTT	GTTCATCATG	TTTCCGTAG	GTAGGTATGT	16560
GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	GTGTGTGTGT	GTGTGTATCT	16620
ATGTATGTAC	GTATGTATGT	ATGTATGTGA	GTGAGATGGG	TTTCGGGGTT	CTATCATGTT	16680
GCCCACGCTG	GTCTCGAACT	CCTGTCCTCA	.AGCAATCCGC	CTGCTGCCT	CGGCCGCCA	16740
CACTGCTGCT	ATTACAGCG	TGAGACGCTG	CGCCTGGCTC	CTTCTACATT	TGCTGCCTG	16800
CCTGCCTGCC	TGCCTGCCTA	TCATCGCT	TCTTTTATGTT	ACGGATGTG	TCTCGCTTTA	16860
TTGTCCATGC	TCTGGCACA	CGTGGCTCT	TTTCAAACCT	CTATGATTAT	TATTATTGTA	16920
GGCGTCATCT	CACGTGTCGA	GGTGATCTCG	AACTTTAGG	CTCCAGAGAT	CCTCCCGCAT	16980
CGGCCTCCCG	GAGTGTGTG	ATGACACGCG	TGGGACCGT	ACGCTCTGGT	CGTGTGTTGTC	17040
GTGGGTCGGT	TCTTCCGTT	TTAATACGG	GGACTGCGAA	CGAAGAAAAT	TTTCAGACGC	17100
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GAATATCGCT	TCTTGGGGG	AGGGGCTTC	TTAGGCCATC	ACCGCTTGCG	GGACTACCTC	31560
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GTGCTGGATG	TATCCTGTCA	AGAGACCTGA	GCCTGACACC	GTCGAATTAA	ACACCTTGAC	31680
TGGCTTTGTG	TGTTTGTGTTG	TTTCTGAGAT	GGAGTCTTGC	TCTGTCCCCC	AGGCTGGAGT	31740
GCAGTGGCGT	GATCTCAGCT	CACTGGAAC	TCTGCCCTCT	GGGITCAAGT	GATTCTCTG	31800
TCTCAGCGCC	ACCATGGCCG	GCTCATTTTT	TTTTTTTTT	TTTTTGGTAG	ACACGGGGTT	31860
TCACCCCTCTT	TCATTGGTTT	TCACTGGAGA	TTCTAGATT	GAGCCACACC	TCATTCCGTG	31920
CCACAGAGAG	ACTTCTTTT	TTTTTTTTT	TTTTTAAGCG	CAACGCAACA	TGTCTGCC	31980
ATTTGAGTGG	CTTCCTATAT	CATTATAATT	GTGTTATAGA	TGAAGAAACG	GTATTAAACA	32040
CTGTGCTAAT	GATAGTAAA	GTGAAGACAA	AAGAAGGCT	ATCTATTTTG	TGGTTAGAAT	32100
AAAGTTGCTC	AGTATTTAGA	AGTACCTAA	ATACGTCAGC	ATTTACACTC	TTCTCTAGTAA	32160
AAGCTGGCCG	ATCTGAATAA	TCTCTTTTA	AACAAACACA	ATTTTGATA	GGGTTAAGAT	32220
TTTTTAAGA	ATGCGACTCC	TGAAAATAG	CTGAACAGAC	GATACACATT	AAAAAAATA	32280
ACAACACAAG	GATCAACCAG	ACTTGGGAAA	AAATCGAAA	CCACACAAGT	CTTATGAAGA	32340
ACTGAGTTCT	AAAAATAGGA	CGGAGAACGT	AGCTATCGGA	AGAGAAGGCA	GTATTGGCAA	32400
GTTGATTGTT	ACGTTGGTCA	GCAGTAGCTG	GCACATATCTT	TTTGGCCATC	TTTCGGGCAA	32460
TGTAACACT	ACAGCAAAAT	GAGATATGAT	CCATTAAACA	ACATATTCCG	AAATCAAAA	32520
GTGTTTCAGT	AATAATAATGC	TTCAGATTTA	GAAGCAAATC	AAATGATAGA	ACTCCACTGC	32580
TGTAATAAGT	CACCCCAAG	ATCACCGTAT	CTGACAAAAT	AACTACCACA	GGGTTATGAC	32640
TTCAGAATCA	TACTTTCTTC	TTGATATTAA	CTTATGTATT	TATTTTTTTT	AATTATTTTC	32700
TCTTGAGACG	CGTCTCGTC	TGTCGCCAG	GCTGGAGTGC	GATGGTGTGA	TCTCGGCTCA	32760
CTGCAACCGC	CACCTCCCTG	GGITCAAGCG	ATTCTCTGC	CTCAGCCTCC	CGAGTAGCTG	32820
GGACTACAGG	TGCCCGCCAC	CACGCCAGC	TAATCTTAT	ACTTTTAATA	GAGAAGGGGT	32880
TTCACCGTGT	CGGCCCCGAT	GGTCTCGATC	TCTTGACCTC	GTGACCCGCC	CGCCTCGGCC	32940
TCCCCAAAGTG	CTGGGATGAC	AGGCGTGGAGC	CACTGAGCCC	GGCCTTCTCT	TGACGTTTAA	33000
ACTATGAACT	CAGTCCAGAG	AAACGCAATA	AATGTCAACG	GTGAGGATGG	TGTTGAGGCA	33060
GAAGTAGGAC	CACACTTTT	CCTATCTTAT	TCAGTTGATA	ACAATATGAC	CTAGGTAGTA	33120
ATTCCTATG	TGCCTACTTA	TACACGAGTA	CAAAAGAGTA	AAACAGAGAG	ACTGCTAAAT	33180
TAAAGGGTAC	GTGAAGTTCT	TCATAGTAAC	TCCGTAAC	GGAAACACTGT	CAAAAAGCAG	33240
CAGCTAGTGA	ATTGTTTCA	TGTATTTTTC	TATTATCCAA	TAAGTGAACT	ATGCTATTCC	33300
TTTCCAGTCT	CCCAAGCACT	TCTGTCCCC	ATCACCACTT	CGGTGCTCGA	AGAAAAAGTA	33360
AGCAAATCAA	GGAACACAAG	CTAAAGAAAC	ACACACACAA	ACAAAGAGCA	ACTACAGCGT	33420
CTGAAAAGT	TTGCTAGAAG	ACTGAAAATG	TTGAGTATAA	GGATCTGGTA	TTCTACGATC	33480
ATGAGTTCAC	TTTCAGAGTTT	GTTCAAGACA	TACGTTTCGT	AAGGAAACAT	TTTAGTTAGA	33540
AGTTATTTCAG	CAGTAGGTAC	CATCCCTAAG	TATTTTCAC	CAAATCCGTG	ACAATAAAGA	33600
GCTATCTAAC	CAGAAAAATT	AGCGAGTACG	GGCACCATCC	ATAGGGCTTT	GTCTTACGC	33660
TTCATTAGCA	CTTACCATGC	CTTACAAATG	CTAGGATTGA	CCCTGATAGC	ATTTCGAAA	33720
CAAGCTAATG	CTTGTCCAG	TTCTTCAGT	AAGACAACTC	ACGCCCTAAT	GCGCTATAGG	33780
CATAAGCATC	ATTGGATCC	ACTTCGAGAG	TTCTCTGGAA	GAATTGAATC	GCAATATCGT	33840
GTTCCCGTTT	GCAGACCGAA	ACAGTTTCCC	TGCAGCACAC	CAGGCCCTCG	GCTGGCGAAT	33900
TTTATCCAT	GTCTGTGAAG	TCTTTGGACA	GAACGTAAAAG	AGCAACCTCT	TTCCGGAGGAT	33960
GCCAAAGTGT	TGTAGAGTAG	ATCTCCATGC	CTTCGACTCT	GTAATTCTCA	ATCCTCCTAA	34020
CCTCTGAGAA	TTGTCTTTCA	GCTTGGTGG	ACTCTGAAAG	TTTACAATAG	GCCNTTCCG	34080
ATTTGGCACA	GTACCCAACC	GGTATTGCGAG	TGGTGAGAAG	CTAGATGGCT	CAAGATGCTG	34140
ATAGCTTCTT	TGCCGTGGTA	AGAACACAAA	GCTAAATAAC	CTTTCCCCCT	TTCAACGAAGA	34200

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AGGCTCATCA	AGCCTCCGC	TGCTGCTTT	TGTAGATTAA	AAGCCTGAAT	CTGAGGCAGC	34260
ATTGCGCTA	TTTTCCTTC	TGAAATGACG	GAAGAGTCCA	ATTTGTCAC	TTCCAGGCTA	34320
TCACTTATGT	TCGGGAGT	TATTGCTCCT	TTATTAGTT	TACTTTGGT	TCTCTGTTT	34380
GGGATTITAG	GTGGAAACTT	CATTTTAAC	TTTCTCTAA	TTCTCTCGG	TTGTGGAGCT	34440
GTCACTAGTC	AAGAGTCGTG	AATTCTTCG	AGGNCGGTGC	ATTGGGGGA	GATGCCATAG	34500
TGGGGCTCAA	TACCTGAGGT	GTGCCCCCTG	TCGGCGGACC	AGAACTTTG	TTTTTGCAA	34560
GGACTGGAGT	TACCTTCGG	CTCTTCCCC	TCTCGAGAA	GACAGACGGT	TTCCGGTTT	34620
GGCCGATTCT	GGCAACAGGC	TTTCTGAAG	GGGCTCCGGT	GGATGGCACG	TCAGTGACAG	34680
ACGGTGTCTC	ATACCAGTGC	AGTTTGTCA	ATAGGGTCCG	TCTCCGGGAC	TTGGGGTTTC	34740
TAATGGCAAA	ATGCCAACAC	TTGGGGTTAA	TGGACTAAC	GCTGCTGGTC	CTCCTAATAA	34800
ACTTCGACCA	GTTTTTGGTT	TATGTTGAAC	CTGTTAGAT	CATATGGAAG	TTCTCTTCCC	34860
CAGTGGGACA	GTATCAGGTG	AAAGGACAGC	TGAATCGATA	GAAGACACTG	GGGAGTCTGT	34920
ATTCAAGGAG	TACTTTGAAT	TGGAAGATTG	TAAATTCCAT	CCGTTTCAATT	CGACGGGTGC	34980
CTGGGGTGT	TCCGTAAGAA	CGGTCTCGGG	CTGTCGTGTA	CATAAAACTAG	GACGAGGTCC	35040
AAGTGTGTG	GCGCAACACT	TGGACAGGCA	GTTGCTAAAG	CTCTCTAGAG	AGGTGAATCA	35100
AAATGTTGG	TCAGGATCTG	GCTTTCCCC	CCTATTTCAC	ATCATGATT	AAAGGGACAC	35160
CAGAGGAAAG	GATTTCAACG	AAGGCTCTTT	TGGTCACATT	CTGATCCTT	GGTAAGCCGA	35220
TCTGCTTGC	AATATACATG	TCCCAGCAGT	GGAAAGGGAA	AGCGAGCTGA	ATCACCAAAC	35280
TCAGGAACGA	TAATATCATC	GTGGCTTTTC	TGCTTATGAA	ACACTCCACC	CGATAAGATT	35340
TGATCCCCTT	CTGCAAGCTT	GCTGAGATCA	ACACAAACATT	TGCGAACAG	GCATTTGCAT	35400
TGCGGGTAG	TACAACCTGTG	TCCTTCAAG	AGTCTATATG	TTTTATAGGC	TTTCCTGAG	35460
CGGTAAGAAC	AGGTGCGGAG	TAAGAACAAAG	GCTTCTCTG	AGTGTACTTC	TGCTATAAGG	35520
CGTTCTGCGG	GGGAAACCGC	ATCTCGGTAG	GCATAGTGGT	TTAGTGTCTG	CCATATAGCA	35580
GCCTGGACGG	GTCCCTGCAAG	CACCGCCATC	CTCGAGGCTC	AGGCCCCACTT	TCTGCAGTGC	35640
CACAGGACCAC	CCCCCCCCCCC	CATAGCGGCT	CCGGCCCGGC	CAGCCCCGGC	TCATTTAAAG	35700
GCACCAAGCCG	CCGTTACCGG	GGGATGGGGG	AGTCCGAGAC	AGAATGACTT	CTTTATCCTG	35760
CTGACTCTGG	AAAGCCCGGC	GCCTTGTGAT	CCATTGAAA	CCGAGAGTCA	CCTCGTGT	35820
AGAACACGGA	TCCACTCCCA	AGTTCACTGG	GGGGATGTGA	GGGGGTGTGGC	AGGTAGGACG	35880
AAGGACTCTC	TTCCCTCTGA	TTGGTCTGC	ACAGTGGGGC	CTAGGGCTGG	AGCTCTCTCC	35940
GTGCGGACCG	CTGACTCCCT	CTACCTTGGG	TTCCCTCGGC	CCCACCCCTGG	AACGCCGGGC	36000
CTTGGCAGAT	TCTGGCCCTT	TCTGGCCCTT	CAGTCGCTGT	CAGAAACCCC	ATCTCATGCT	36060
CGGATGCCCG	GAGTGAATGT	GGCTCGCACC	TCTCCGAAA	CATTGGAAT	CTCTCCTCTA	36120
CGCGGGGCCA	CCTGAAACCA	CAGGAGCTCG	GGACACACGT	GCCTTCGGGA	GAGAATGCTG	36180
AGAGTCTCTC	GCCGACTCTC	TCTTGACTTG	AGTTCTCGT	GGGTGCGTGG	TTAAGACGTA	36240
GTGAGACCAG	ATGTATTAAC	TCAGGCCGGG	TGCTGGTGGC	TCACGCTGT	AACCCPACA	36300
CTTGGGAGG	CCGAGGCCGT	AGGATCCCTC	GAGGAATCGC	CTAACCCCTGG	GGAGGTTGAG	36360
GTTGCACTGA	GTGAGCCATA	GTTGTGTAC	TGTGCTCCAG	TCTGGCGAAG	AGACAGAAATG	36420
AGGCCCTGCC	ACAGGCAGGC	AGGCAGGCAG	GCAGGCAGAA	AGACAACAGC	TGTATTATGT	36480
TCTTCTCAGG	GTAGGAAGCA	AAAATAACAG	AATAACGAC	TIAATTAAATT	TTTTTTTTTT	36540
CCTTCGGACG	GAGTTTCACT	CTTGGTGGCC	ACGCTGGAGT	GCAGTGGCAC	CATCTCGGCT	36600
CACCGCAACC	TCCACCTCCC	GCCTCAAGC	GATTCTCTG	CTTCAGCCTC	CTGAGTAGCT	36660
GGGATTACAG	GGAGGAGGCCA	CCACACCCAG	CTGATTTTGT	ATTGTTAGTA	GAGACGGCAT	36720
TTCTCCATGT	GGGTCAAGGT	GGTCTCGAAC	TGGCGACCCC	AGTGGATCTG	CCCGCCCCGG	36780
CCTCCCAAAG	TGCTGGGGTG	ACAGGCGTGA	GCCATCGTGA	CTGGCCGGCT	ACGTTTATT	36840
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TACATTATTT	TATTTATTTA	TTTATTTACT	TATTTATTTA	TTTCGAGAC	AGACTCTCGC	36960
TCTGCTGCC	AGGCTGGAGT	GCAGCGGCGT	GATCTCGGCT	CACTGCAACG	TCCGCCCTCCC	37020
GGGTTACAGC	CATTCTCTG	CCTCAGCCTC	CCAAGTAGCT	GGGACTACAG	GCGCCCGCCA	37080
CCGTGCCCGG	CTAACCTTT	GTATTTGAG	AGAGATGGG	TTTCACTGT	GGTAGCCAGG	37140
ATGGTCTCGA	TCTCTGACC	CCGTGATCCG	TCCACCTCGG	CCTCCCAAAG	TGCTGGGATG	37200
ACAGGGCTGA	GCCACCGGCC	CCGGCCTATT	TATCTATTTA	TTAACCTTGA	GTCCAGGTTA	37260
TGAAACAGT	TAGTTTTGT	AATTTTTTT	TTTTTTTTT	TTTTTTGAGA	CGAGGTTTCA	37320
CCGTGTTGCC	AAGGCTTGG	CCGAGGGATC	CACCGGCCCT	CCGCCTCCCA	AAAGTGCAGG	37380
GATGACAGGC	GCGAGCCTAC	CGCGCCCCGG	CCCCCCTTT	CCCCCTCCCC	CGCTTGTCTT	37440
CCCGACAGAC	AGTTTACCGG	CAGAGCGTTT	GGCTGGCGTG	CTTAAACTCA	TTCTAAATAG	37500
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CTATCACAGG	ACCGTACACG	TAAGGAGGAG	AAAAATCGTA	ACGTTCAAAG	TCAGTCATT	37620
TGTGATACAG	AAATACACGG	ATTACCCCAA	AACACAGAAA	CAAGTCCTT	AGAAAATGGCC	37680
TTAGCCCTGG	TGTCCGTGCC	AGTGTATTCTT	TTCGGTTGG	ACCTTGACTG	AGAGGATTCC	37740
CAGTCGGTCT	CTCGTCTCTG	GACGGAAAGTT	CCAGATGATC	CGATGGGTGG	GGGACTTAGG	37800
CTGCGTCCCC	CCAGGAGGCC	TGGTCGATTA	GTTGTGGGGA	TCGCCTTGG	GGGCAGGGTG	37860

ACCCACTGTG	CTGTGGGAGC	CTCCATCCTT	CCCCCCACCC	CCTCCCCAGG	GGGATCCCAA	37920
TTCATTCCGG	GCTGACACGC	TCACTGGCAG	GCGTCGGCA	TCACCTAGCG	GTCACTGTTA	37980
CTCTGAAAAC	GGAGGCCTCA	CAGAGGAAGG	GAGCACCAAGG	CCGCCTGCAG	ACAGCCTGGG	38040
GCAACTGTG	CTTCTCCACC	GCCCCCGCCC	CCACCTCCAA	GTTCCTCCCT	CCCTTGTGTC	38100
CTAGGAAATC	GCCACTTGTG	CGACCGGGTC	TGATTGACCT	TTGATCAGGC	AAAAACGAAC	38160
AAACAGATAA	ATAAATAAAA	TAACACAAAA	GTAACTAATC	AAATAAAATA	AGTCAATACA	38220
ACCCATTACA	ATACAATAAG	ATACGATACG	ATAGGATGCG	ATAGGATAACG	ATAGGATAACA	38280
ATACAAATAGG	ATACGATACA	ATACAATACA	ATACAATACA	ATACAATACA	ATACAATACA	38340
ATACAATACA	ATACAATACG	CCGGGCGCGG	TGGCTCATGC	CTGTCATCCC	GTCACTTTGG	38400
GATGCCGAGG	TGGACGCATC	ACCTGAAGTC	GGGAGTTGGA	GACAAGCCCG	ACCAACATGG	38460
AGAAATCCCG	TCTCAATTGT	AAATACAAAA	CTAGCCGGC	GCGGTGGCAC	ATGCTTATAA	38520
TCCCAGCTGC	TAGGAAGGCT	GAGGCAGGAG	AATCGCTTGA	ACCTGGGAAG	CGGAGGTTGC	38580
AGTGAGCCGA	GATTGCGCCA	TCGCACTCCA	GTCTGAGCAA	CAAGAGCGAA	ACTCCGTCTC	38640
AAAAATAAAT	ACATAAATAA	ATACATACAT	ACATACATAC	ATACATACAT	ACATACATAC	38700
ATAAAATAAA	ATAAATAAAT	AAAATAAAAT	AAATAATGG	GCCCTGCGCG	GTGGCTCAAG	38760
CCTGTATCC	CCTCACTTTG	GGAGGCCAAC	GCCGGTGGAT	CAAGAGGCCG	TCAGACCAAC	38820
AGGGCAGTA	TTGTGAAACC	CGGTCTCTAC	TCACAATACA	CAACATTAGC	CGGGCGCTGT	38880
GCTGTGCTGT	ACTGTCTGT	ATCCCAGCTA	CTCGGGAGGC	CGAGCTGAGG	CAGGAGAACATC	38940
GCTTGAACCT	GGGGAGGCGGA	GTTGCACTGC	AGCCGAGATC	GCGCCACTGC	AACCCAGCCT	39000
GGGCGACAGA	GCGAGACTCC	GTCCTCCAAA	AATGAAATG	AAAATGAAAC	GCAACAAAAT	39060
AATTAAAAAG	TGAGTTCTG	GGGAAAAAGA	AGAAAAAGAA	AGAAAAAGAA	ACAAACAAAAC	39120
AGAACAAACCC	CACCGTGACA	TACACGTACG	CTTCTCGCCT	TTGAGGCTC	CAAACACGTT	39180
AGGAATTATG	CGTGATTTCT	TTTTTTAACT	TCATTITATG	TTATTATCAT	GATTGATGTT	39240
TCGAGACGGA	GTCTCGGAGG	CCCGCCCTCC	CTGGTTGCC	AGACAACCCC	GGGAGACAGA	39300
CCCTGGCTGG	GGCCGATTGT	TCTTCTCCTT	GGTCAGGGGT	TTCTTGTCT	TTCTCGTGT	39360
CTTAAACCCG	CGTGGACTCT	TCCGCCTCGG	GTTTGACAGA	TGGCAGCTCC	ACTTTAGGCC	39420
TTGTTGTTGT	TGGGGACTTT	CCTGATTCTC	CCCAGATGTA	GTGAAAGCAG	GTAGATTGCC	39480
TTGCTTGGCC	TTGCTTGGCC	TTGCTTCTT	TTTCTTCTT	TCCTTCTTTA	TTACTTTCTC	39540
TTTTCTTCT	TCTTCTTCTT	CTTTTTTTG	AGACAGAGT	TCACTTCTGT	TGCCCAAGGCT	39600
AGAGGGCAAT	GGCGCGATCT	CGGCTCACCG	CACCCCTCCG	CTCCCAGGT	CAAGCGATT	39660
TCCTGCCTCA	GCCTCCTGAT	TAGCTGGAT	TACAGGCATG	GGGCCACCGTG	CTGGCTGATG	39720
TTTGTACTTT	TAGTAGAGAC	GGTGTTCCTC	CATGTTGGTC	AGGCTGGTCT	CCCACTCCCA	39780
ACCTCAGGTG	GTCCGCTGTC	CTTAGCCTCC	CAAAGTGCTG	GGATGACAGG	CGTGAACCG	39840
CGCCCGACCT	CTCTCTCTCT	CTCTCTCTCT	CTCGCTCGCT	TGCTTGTCTG	CTTTCTGTCT	39900
TTCTTGTCTT	CCCGTTTCT	TGCTTCTTTT	CTTTCTTCG	TTTCTTCTCAT	GCTTGTCTTC	39960
TTGCTTGTCT	GCTTGTCTTC	GTGCTTCTTT	GCTTTCTCT	TTTCTTCTT	TCTTCTTCTC	40020
TTTCTTGTCT	TTGTTTCTT	CTTGCTTGT	TTCTTGTCTG	CTTGCTTGT	TCGTGCTTT	40080
CTTGCTTCTC	TGTTTCTT	CTTCTTCTT	TTCTTCTTCT	TTCTTCTT	TTGCTTCTCT	40140
GCTTGTCTG	TTTCGTGCTT	TCTTGTCTTC	TCGATTCTT	TCTTCTTCTT	GTTTCTTCTC	40200
TGCTTGTCTT	CTTGCTTGT	TGCTTCTTCG	CTTCTTGTCT	TCCTTCTTCT	TTTCTTCTT	40260
TCTTCTTCTT	GTTCCTTCT	TGCTTCTT	CTTGCTTGT	TCCTTCTTCT	TTTCTTCTT	40320
TCTCGATTTC	TTTCTTCTT	TTGTTTCTT	CCTGCTTGT	TTCTTGTCTG	ATTGCTTCTG	40380
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TTTCTTGTCT	TCTTGTCTG	TTGCTTCTCGT	GCTTTCTTGT	TTTCTTGTCT	TCTTCTTCTT	40500
GTTTCTTCT	TGCTTGTCT	CTTGCTTCTCT	TGTTTCTTGT	CTTCTTGTCT	TGCTTGTCTT	40560
CGTGCTTCT	TTCTTGTCT	CTTCTTCTTC	TTTCTTCTCT	TTCTTGTCTT	TTCTTGTCTT	40620
CTTTTCTTCT	ATCATCATCT	TTCTTCTT	CCTTCTTCTC	TTTCTTCTT	TCTATCTTTC	40680
TTTCTTGTCT	TCTTCTTCTT	TTTCTTCTT	TCTTCTTCTG	TCTTCTTCTT	TCTATCTTTC	40740
TTCACTCTG	TTTCCACGGC	TAGAGTGCAA	TGGCGCATC	TTGGCTCACC	GCACCTTCCG	40800
CCTCCGGGGT	TCGAGCGCTT	CTCCCTGCC	CAGCCTCCCG	ATTAGGGGGG	ATTGACAGGG	40860
AGGCACCCCC	ACGCCTGGCT	TGGCTGATGT	TTGTGTTTT	AGTAGGCACG	CCGTGTCTCT	40920
CCATGTTGCT	CAGGCTGGTC	TCCAACCCC	GACCTCTGT	GATGCGCCCA	CCTCGGCCTC	40980
TCGAAGTGCT	GGGATGACGG	GC GTGACGAC	CGTGCCCGC	CTGTTGACTC	ATTCGCTTT	41040
TTTATTTCTT	TCGTTTCCAC	GC GTT TACTT	ATATGTATA	ATGTAACAGT	TTCTGTACGC	41100
TTATATGCAA	ACAACGACAA	CGTGTATCTC	TGCATTGAAT	ACTCTTGCCT	ATGGTAAATA	41160
CGTATCGGTT	GTATGGAAAT	AGACTTCTGT	ATGATAGATG	TAGGTGTCTG	TGTTATACAA	41220
ATAAAATACAC	ATCGCTCTAT	AAAGAAGGGAA	TCGTCGATAA	AGACGTTTAT	TTTACGTATG	41280
AAAAGCGTCG	TATTTATGTG	TGTAATGAA	CCGAGCGTAC	GTAGTTATCT	CTGTTTCTT	41340
TCTTCCTCTC	CTTCGTGTTT	TTCTTCTTC	CTTTCTTCT	TTCTTCTCTT	CTT TAGGTTT	41400
TTCTTCCTCT	CTTCCTTCTC	TTCTTCTCT	CTTTCTGTCC	TTTTTCTTCTT	CGTGTCTTAT	41460
TTCTCTTCTG	TTCCCTGTGT	TTCTTCTT	TTTCTTCTCT	CTCTGTCTCT	TTTCTCTTCTC	41520

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TTTCCCTTCGT	TTCTTTCTC	ATTCTTTCTC	TCTTTTCTCGT	TGTTTCTTTC	CTTCCCGTCT	41580
GTCTTTAAA	AAATTGGAGT	GTTTCAGAAG	TTTACTTTGT	GTATCTACGT	TTTCTAAATT	41640
GTCTCTCTT	TCTCCATTT	CTTCCCTCCCT	CCCTCCCTCC	CTCCCTGTC	CCTTCCCTCC	41700
CTCCCTCCCT	TTCGCCATCT	GTCTCTTTTC	CCCACCTCCCC	TCCCCCCCCTC	TGTCTCTGCG	41760
TGGATTCCGG	AAGAGCCTAC	CGATTCTGCC	TCTCCGTGTG	TCTGCAGCGA	CCCCCGGACCC	41820
GAGTCCTTGT	GTGTTCTTC	TCCCTCCCTC	CCTCCCTCCC	TCCCTCCCTC	CCTCCCTGCT	41880
TCCGAGAGGC	ATCTCCAGAG	ACCGCGCCGT	GGGTTGTCTT	CTGACTCTGT	CGCGGTGAG	41940
GCAGAGACGC	GTTTTGGCA	CCGTTTGTGT	GGGGTTGGGG	CAGAGGGGCT	GCGTTTTCGG	42000
CCTCGGGAAG	AGCTTCTCGA	CTCACGGTTT	CGCTTCTGCC	GTCCACGGGC	CGCCCTGCCA	42060
GCCGGATCTG	TCTCGCTGAC	GTCCCGGCCG	GTTGTCGGGC	TCCATCTGGC	GGCCGCTTTG	42120
AGATCGTGCT	CTCGGCTTCC	GGAGCTGCG	TGGCAGCTGC	CGAGGGAGGG	GACCCTCCCC	42180
GCTGTGAGCT	AGGCAGAGCT	CCGAAAGCC	CGCGGTGTC	AGCCCCGGCTG	GCCCCGGTGGC	42240
GCCAGAGCTG	TGGCCGGTGC	CTTGTGAGTC	ACAGCTCTGG	CGTGCAGGTT	TATGTGGGGG	42300
AGAGGCTGTC	GCTGCGCTTC	TGGGCCCGCG	GCAGGGCGTGG	GGCTGCCCGG	GCCGGTGCAC	42360
CAGCGCGCCG	TAGCTCCCGA	GGCCCGAGCC	GCGACCCGGC	GGACCCGCCG	CGCGTGGCGG	42420
AGGCTGGGA	CGCCCTTCCC	GGCCCGGTGCG	CGGTCCGCTC	ATCCTGGCCG	TCTGAGGCAG	42480
CGGCCGAATT	CGTTTCCGAG	ATCCCCGTGG	GGAGCCGGGG	ACCGTCCCGC	CCCCGTCCCC	42540
CGGGTGCAGG	GGAGCGGTCC	CCGGGCCGGG	CCGCGGTCCC	TCTGCCGCGA	TCCTTTCTGG	42600
CGAGTCCCCG	TGGCCAGTCG	GAGAGCGCTC	CCTGAGCCGG	TGCAGGGCCG	GAGGTCGCGC	42660
TGGCCGGCC	TGGGTCCCCC	GTGTGTCCCG	GTCGTAGGAG	GGGCCGGCCG	AAAATGCTTC	42720
CGGCTCCC	TCTGGAGACA	CGGGCCGGCC	CCTGCGTGTG	GCCAGGGCGG	CCGGGAGGGC	42780
TCCCCGGCCC	GGCGCTGTCC	CCGCGTGTGT	CCTTGGGTTG	ACCAGAGGGA	CCCCGGGCGC	42840
TCCGTGTGTG	GCTGCGATGG	TGGCGTTTT	GGGGACAGGT	GTCCGTGTCC	GTGTCGCGCG	42900
TCGCCTGGGC	CGGGCGCGTG	GTCGGTGACG	CGACCTCCCG	GCCCCGGGGGG	AGGTATATCT	42960
TTCGCTCCGA	GTGCGCAATT	TTGGGCCGCC	GGGTTATAT			42999

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTCCCCGGCG	GCCCCCGTGT	TCGCCGTTCC	CGTGGCGCGG	ACAATGCGGT	TGTGCGTCCA	60
CGTGTGCGTG	TCCGTGCAGT	GCCGTTGTGG	AGTGCCTCGC	TCTCCTCCCTC	CTCCCCGGCA	120
GCCTTCCCAC	GGTTGGGAC	CACCGGTGAC	CTCGCCCTCT	TCGGGCCTGG	ATCCG	175

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 755 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTCTGGTGG	GAATTGTTGA	CCTCGCTCTC	GGGTGCGGCC	TTTGGGAAAC	GGCGGGGTCG	60
GTCGTGCCG	GCGCCGGACG	TGTGTCGGGG	CCCACTTCCC	GCTCGAGGGT	GGCGGTGGCG	120
GCGGCCTGG	TAGTCTCCG	TGTTGCGTCT	TCCCAGGCTC	TTGGGGGGGG	TGCCGTCGTT	180
TTCGGGGCCG	GCGTTGCTTG	GCTTACGCAG	GCTTGTTTG	GGACTGCCTC	AGGAGTCGTG	240
GGCGGTGTGA	TTCGGCCTCG	TTTGCGCTG	CGTCTGCCTG	CTTGCGCTCG	GGTTTGCTTG	300
GTTCTGTCT	CGGGAGCGGT	GGTTTTTTT	TTTTCGGGT	CCCGGGGAGA	GGGGTTTTTC	360
CGGGGGACGT	TCCCCTCGCC	CCCTGCGCC	GGTGGGTTT	CGTTTCGGGC	TGTGTTCGTT	420
TCCCCCTCCC	CGTTTCGGCG	TCGGTTCTCC	CCGGTCGGTC	GGCCCTCTCC	CCGGTCGGTC	480
GCCCCGCCGT	GCTGCCGAC	CCCCCCTCTC	GGGGGGGATG	CCCGGGCACG	CACCGGTCCG	540
GGCGGCCACT	GTGGTCCGGG	AGCTGCTCGG	CAGGCGGGTG	AGCCAGTTGG	AGGGGCGTCA	600
TGCCCCCGCG	GGCTCCCGTG	GCCGACGCGG	CGTGTCTTT	GGGGGGGCCT	GTGCGTGCAG	660
GAAGGCTGCG	CACGTTGTCG	GTCCTTGCAG	GGGAAAGAGG	CTTTTTTTTT	TTAGGGGGTC	720
GTCCTTCGTC	GTCGGTCCGG	CGGTGGATCC	GGCCT			755

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 463 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCGAGGTG	CGTCTGCGGG	TTGGGGCTCG	TCCGGCCCCG	TCGTCCTCCG	GGAAGGCCTT	60
TAGCGGGTAC	CGTCGCCCG	CCGAGGTGGG	CGCACGTCGG	TGAGATAACC	CCGAGCGTGT	120
TTCCTGGTGT	TGGCGGCCGG	GGCTCCGGTC	GATGTCCTCC	CCTCCCCCTC	TCCCCGAGGC	180
CAGGTCAAGCC	TCCGGCTGTG	GGCTTCGTG	GCCGTCCTCCC	CCCCCCTCAC	GTCCCTCGCG	240
AGCGAGCCCG	TCCGTTGAC	CTTCCTTCGG	CCTTCCCCCC	ATCTTTCCGC	GCTCCGTTGG	300
CCCCGGGGTT	TTCACGGCGC	CCCCCACGCT	CCTCCGCCTC	TCCGCCCCGTG	GTTGGACGC	360
CTGGTTCCGG	TCTCCCCGCC	AAACCCCGGT	TGGGTTGGTC	TCCGGCCCCCG	GCTTGCTCTT	420
CGGGTCTCCC	AACCCCCGGC	CGGAAGGGTT	CGGGGGTTCC	GGG		463

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 378 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGATTCTTCA	GGATTGAAAC	CCAAACCGGT	TCAGTTTCCT	TTCCGGCTCC	GGCCGGGGGG	60
GGCGGGCCCCG	GGCGGTTTGG	TGAGTTAGAT	AACCTCGGGC	CGATCGCACG	CCCCCCGTGG	120
CGGCGACGAC	CAATTGAAAC	GTCTGCCCTA	TCAACTTTG	ATGGTAGTCG	ATGTGCCTAC	180
CATGGTGACC	ACGGGGTGAAC	GGGAATCAGG	GTTCGATTCC	GGAGAGGGAG	CCTGAGAAC	240

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GGCTACCACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCACTCCCGA CCCGGGGAGG	300
TAGTGACGAA AAATAACAAT ACAGGACTCT TTCGAGGCC TGTAATTGGA ATGAGTCCAC	360
TTTAAATCCT TTAAGCAG	378

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 378 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCCATTGG AGGGCAAGTC TGGTGCCAGC AGCCGCGGTA ATTCCAGCTC CAATAGCGTA	60
TATTAAAGTT GCTGCAGTTA AAAAGCTCGT AGTTGGATCT TGGGAGCGGG CGGGCGGTCC	120
GCCGCGAGGC GAGTCACCGC CCGTCCCCGC CCCTTGCCTC TCAGCGCCCC CTCGATGCTC	180
TTAGCTGAGT TGTCCCAGCGG GGCCCAGAAC GTTTACTTTG AAAAAATTAG AGTTGTTCA	240
AAGCAGGCCG GAGCCGCCTG GATAACGCCA GCTAGGAAAT AATGGAATAG GACCGCGGTT	300
CCTATTTGT TTGGTTTCG GAACTGAGCC CATGATTAAG GGAAACGGCC GGGGGCATTC	360
CCTTATTGCG CCCCCCTA	378

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 719 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGATCTTTCC CGCTCCCCGT TCCTCCCGGC CCCTCCACCC GCGCGTCTCC CCCCTTCTTT	60
TCCCCTCTCC GGAGGGGGGG GAGGTGGGG CGCGTGGCG GGGTCGGGG TGGGGTGGC	120
GGGGGACCGC CCCCAGGGCG CAAAAGGCCG CCGCCGGCG CACTTCAACC GTAGCGGTGC	180
GCCCGCACCG GCTACGAGAC GGCTGGGAAG GCCCAGCGG GAATGTGGCT CGGGGGGGGC	240
GGCGCGTCTC AGGGCGCGCC GAAACACCTC ACCCGAGTG TTACAGCCTC CGGGCGCGC	300
TTTCGCGGAA TCCCGGGGCC GAGGGGAAGC CCGATAACCG TCGCCGCGT TTTCCCTCC	360
CCCCGTCCGC CTCCCGGGCG GGCGTGGGG TGGGGGCCGG GCGCCCCCTC CCACGCCCCGT	420
GGTTTCTCTC TCTCCCGGTC TCAGCGGTG TGGGGGGGG AGCCCGGTTG GGGGCAGGGC	480
GGACTGTCCT CAGTGCAGCC CGGGCGTCGT CGCGCCGTG GGCAGGGGG GTTCTCTCGG	540
TCACGGCGCC CCCGACGAAG CCGAGCGCAC GGGGTCGGCG GCGATGTGG CTACCCACCC	600
GACCCGTCTT GAAACACGGA CCAAGGAGTC TAACGCGTGC GCGAGTCAGG GGCTCGCACG	660
AAAGCCGCCG TGGCGCAATG AAGGTGAAGG GCCCCGTCCG GGGGCCCGAG GTGGGATCC	719

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 685 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGAGGCCTCT	CCAGTCCGCC	GAGGGCGCAC	CACCGGCCC	TCTCGCCCGC	CGCGTCGGGG	60
AGGTGGAGCA	CGAGCGTACG	CGTTAGGACC	CGAAAGATGG	TGAACATATGC	CTGGGCAGGG	120
CGAACCCAGA	GGAAACTCTG	GTGGAGGTCC	GTAGCGGTCC	TGACGTGCAA	ATCGGTCGTC	180
CGACCTGGGT	ATAGGGCGA	AAGACTAAC	GAACCATCTA	GTAGCTGGTT	CCCTCCGAAG	240
TTTCCCTCAG	GATAGCTGGC	GCTCTCGCAA	CCTTCGGAAAG	CAGTTTATC	CGGGTAAAGG	300
CGGAATGGAT	TAGGAGGTCT	TGGGGCCGGA	AACGATCTCA	AACTATTCT	CAAACTTAA	360
ATGGGTAAGG	AAGCCCGGCT	CGCTGGCGTG	GAGCCGGGCG	TGGAATGCGA	GTGCCTAGTG	420
GGCCACTTTT	GGTAAGCAGA	ACTGGCGCTG	CGGGATGAAC	CGAACGCCGG	GTAAAGCGC	480
CCGATGCCGA	CGCTCATCAG	ACCCAGAAA	AGGTGTTGGT	TGATATAGAC	AGCAGGACGG	540
TGGCCATGGA	AGTCGGAATC	CGCTAAGGAG	TGTGTAACAA	CTCACCTGCC	GAATCAACTA	600
GCCCTGAAAA	TGGATGGCGC	TGGAGCGTCG	GGCCCATACC	CGGCCGTGCG	CGGCAGTCGG	660
AACGGGACGG	GACGGGAGCG	GCCGC				685

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SCURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAGGAATTCC CCTATCCCTA ATCCAGATTG GTG

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(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AAACTGCAGG CCGAGCCACC TCTCTCTGT GTTTG

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(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGGAATTCA C AGAAGAGAGG TGGCTCGGCC TGC

33

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGCCTGCAGG AAGTCATAACC TGGGGAGGTG GCCC

34

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 80 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AAACTGCAGG TTAATTAACC CTAACCCCTAA CCCTAACCCCT AACCCCTAAC CTAACCCCTAA
CCCTAACCCCT AACCCGGGAT60
80

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTGGGCCCTA GGCTTAAGG

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(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCCAGGGTTT TCCCAGTCAC GACGT

25

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCTGCAAGGC GATTAAGTTG GGTAAC

26

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATGTTGTGT GGAATTGTGA GC GGAT

26

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: / /

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGGTTTAAAC AGATCTCTGC A

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WHAT IS CLAIMED:

1. A method for producing an artificial chromosome, comprising:
 - introducing a DNA fragment into a cell, wherein the DNA fragment comprises a selectable marker;
- 5 growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA; and selecting a cell that comprises a satellite artificial chromosome [SATAC].
2. The method of claim 1, wherein the DNA fragment is introduced into or adjacent to an amplifiable region of a chromosome in the cell.
- 10 3. The method of claim 2, wherein the amplifiable region comprises rDNA.
4. The method of claim 2, wherein the amplifiable region comprises heterochromatin.
- 15 5. The method of claim 1 or claim 2, wherein the DNA is introduced into pericentric heterochromatin in a chromosome of the cell.
6. The method of claim 1, wherein the cell is a mammalian cell.
7. The method of claim 1 or claim 2, further comprising,
 - 20 isolating the SATAC.
 8. The method of any of claims 1-7, wherein the DNA fragment comprises a sequence of nucleotides that targets the fragment to the heterochromatic region of a chromosome.
 9. The method of claim 8, wherein the targeting sequence of nucleotides comprises satellite DNA.
 - 25 10. The method of any of claims 1-9, wherein the cell is a human cell.
 11. The method of any of claims 1-5 and 7- 9, wherein the cell is a fish, insect, reptile, amphibian, arachnid, or rodent cell.

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12. A SATAC produced by the method of any of claims 1-11.
13. An isolated substantially pure satellite artificial chromosome [SATAC].
14. The SATAC of claim 13 that is a megachromosome,
- 5 comprising about 50 to about 450 megabases [Mb].
15. The SATAC of claim 13, comprising about 250 to about 400 Mb.
16. The SATAC of claim 13, comprising about 150 to about 200 Mb.
- 10 17. The SATAC of claim 13, comprising about 90 to about 120 Mb.
18. The SATAC of claim 13, comprising about 15 to about 60 Mb.
19. A cell containing an artificial chromosome, wherein the
- 15 artificial chromosome is produced by the method of any of claims 1-11.
20. A cell containing the SATAC of any of claims 12-19.
21. The cell of claim 19 or claim 20 that is a mammalian cell.
22. The method of any of claims 1-11, wherein the SATAC is a megachromosome, and the method further comprises:
- 20 introducing a fragmentation vector, whereby the megachromosomes in the cells are reduced in size, and identifying cells that contain SATACs that are about 15 to about 60 Mb.
23. The method of any of claims 1-11, wherein the SATAC is a
- 25 megachromosome, and the method further comprises, exposing the cells to conditions, whereby cells that contain truncated megachromosomes are produced.
24. The method of claim 23, wherein the conditions are selected from among exposure to X-rays, growth in the presence of an agent that
- 30 destabilizes base pairing in the chromosome.

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25. The method of claim 24, wherein the agent is bromodeoxyuridine.
26. The method of any of claims 22-25, further comprising selecting a cell that comprises a satellite artificial chromosome [SATAC] 5 that comprises about 15 to about 60 Mb.
27. A cell containing an artificial chromosome, wherein the artificial chromosome is produced by the method of any of claims 22-25.
28. The cell of any of claims 19-21, 25-27, wherein the artificial chromosome is a SATAC comprising about 10 to about 60 Mb.
- 10 29. An isolated substantially pure satellite artificial chromosome [SATAC] of claim 13 that comprises about 10 to about 60 Mb.
30. The method of any of claims 1-11 and 22-26, further comprising isolating the SATAC from the cell.
- 15 31. The method of claim 30, wherein isolation is effected by: isolating metaphase chromosomes; distinguishing SATACs from endogenous chromosomes; and separating the SATACs from endogenous chromosomes.
32. The method of claim 31, wherein: the SATACs are distinguished from endogenous chromosomes by 20 staining the chromosomes with DNA sequence-specific dyes; and separation is effected by flow cell sorter.
33. A method for producing an artificial chromosome, comprising:
 - introducing a DNA fragment into a cell, wherein the DNA 25 fragment comprises a selectable marker,
 - growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA,
 - selecting from among those cells, a cell that comprises a *de novo* centromere.

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34. The method of claim 33, further comprising isolating that cell with the chromosome that comprises the *de novo* centromere, and growing the cell under conditions whereby a cell with a sausage chromosome is produced.

5 35. The method of claim 34, further comprising isolating the cell with the sausage chromosome; and growing the cell under conditions whereby a first SATAC is produced.

36. The method of claim 35, wherein the DNA fragment is introduced into or adjacent to an amplifiable region of a chromosome in
10 the cell.

37. The method of claim 36, wherein the amplifiable region comprises rDNA.

38. The method of claim 36, wherein the amplifiable region comprises heterochromatin.

15 39. The method of claim 35 or claim 36, wherein the DNA is introduced into pericentric heterochromatin in a chromosome of the cell.

40. The method of any of claims 33-39, further comprising: introducing a fragmentation vector that is targeted to the first SATAC; growing the cells; and selecting a cell that comprises a second
20 SATAC, wherein the second SATAC is smaller than the first SATAC.

41. The method of claim 40, wherein the selected cell has a dicentric chromosome comprising the *de novo* centromere.

42. The method of claim 40, wherein the selected cell has a formerly dicentric chromosome and a minichromosome comprising the *de*
25 *novo* centromere.

43. The method of claim 40, wherein the selected cell has a formerly dicentric chromosome.

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44. A method for producing an artificial chromosome, comprising introducing a DNA fragment into a cell, wherein the DNA fragment comprises a selectable marker;
- 5 growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA;
- 10 selecting from among those cells a cell that has produced a dicentric chromosome; and growing that cell under selective conditions, whereby a cell that contains a chromosome comprising a heterochromatic arm is produced.
45. The method of claim 44, further comprising selecting the cell with the chromosome comprising the heterochromatic arm and growing it in the presence of an agent that destabilizes the chromosome.
- 15 46. The method of claim 45, further comprising identifying cells that contain a heterochromatic chromosome that is about 50 to about 400 Mb.
- 20 47. The method of any of claims 44-46, wherein the DNA fragment is introduced into or adjacent to an amplifiable region of a chromosome in the cell.
- 25 48. The method of claim 47, wherein the amplifiable region comprises rDNA.
49. The method of claim 47, wherein the amplifiable region comprises heterochromatin.
50. The method of claim 47, wherein the DNA is introduced into pericentric heterochromatin in a chromosome of the cell.
51. A method for producing a transgenic (non-human) animal, comprising introducing a satellite artificial chromosome (SATAC) into an embryonic cell.
- 30 52. The method of claim 51, wherein the embryonic cell is a stem cell.

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53. The method of claim 51, wherein the embryonic cell is in an embryo.

54. The method of any of claims 51-53, wherein the SATAC comprises heterologous DNA that encodes a gene product.

5 55. The method of any of claims 51-54, wherein the SATAC comprises heterologous DNA that encodes a therapeutic product.

56. The method of claim 55, wherein the anti-HIV ribozyme is an anti-gag ribozyme, and the tumor suppressor gene is p53.

10 57. The method of claim 54, wherein the product comprises an antigen that upon expression induces a immunoprotective response against a pathogen in the transgenic (non-human) animal.

58. The method of claim 54, wherein the product comprises a plurality of antigens that upon expression induce an immunoprotective response against a plurality of pathogens.

15 59. The method of any of claims 51-58, wherein the transgenic (non-human) animal is a fish, insect, reptile, amphibian, arachnid or mammal.

60. The method of any of claims 51-59, wherein the SATAC is introduced by cell fusion, lipid-mediated transfection by a carrier system, 20 microinjection, microcell fusion, electroporation, microprojectile, nuclear transfer, bombardment or direct DNA transfer.

61. A transgenic (non-human) animal produced by the method of any of claims 51-60.

62. The transgenic animal that is a fish, insect, reptile, amphibian, 25 arachnid, or mammal.

63. A method of for producing a transgenic plant or animal, comprising:

introducing a DNA fragment into a c II, wherein the DNA fragment comprises a selectable marker;

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growing the cell under selective conditions to produce cells
that have incorporated the DNA fragment into their genomic DNA; and
selecting a cell that comprises a minichromosome that is
about 10 Mb to about 50 Mb that comprises the selectable marker and
5 euchromatin;

isolating the minichromosome and introducing it into a plant
or animal cell.

64. The method of claim 63, wherein: after selecting the cell,
DNA encoding a gene product or products is introduced into the cell, and
10 the cell is grown under selective conditions, whereby cells comprising
minichromosomes comprising the DNA encoding the gene product(s)
are produced.

65. The method of claim 64, wherein: after selecting the
cell,DNA encoding a gene product or products is introduced into the cell,
15 and the cell is grown under selective conditions, whereby cells comprising
SATAcs that comprise the DNA encoding the gene product(s) are
produced.

66. A method for cloning a centromere from an animal or plant,
comprising:

20 preparing a library of DNA fragments that comprise the
genome of the plant or animal;

introducing each of the fragments into mammalian satellite
artificial chromosomes [SATAcs], wherein:

each SATAC comprises a centromere from a different
25 species from the selected plant or animal, and a selectable marker;

introducing each of the SATAcs into the cells and growing
the cells under selective conditions;

identifying cells that have a SATAC; and

selecting from among those cells any that have a SATAC comprising a centromere that differs from the centromeres in the original SATAC.

67. A cell line having the identifying characteristics of any of
5 TF1004G19C5, 19C5xHa4, H1D3 and G3D5, which have been deposited at the ECACC under Accession Nos. 96040926, 96040927, 96040929, and 96040928, respectively.

68. A cell line, comprising a megachromosome that comprises about 50-400 Mb.

10 69. A cell line of claim 68, wherein the megachromosome comprises 250 to about 400 Mb.

70. A cell line of claim 68, wherein the megachromosome comprises about 150 to about 200 Mb.

15 71. A cell line of claim 68, wherein the megachromosome comprises about 90 to about 120 Mb.

72. A cell line of claim 68, wherein the megachromosome comprises about 60 to about 100 Mb.

73. A method for gene therapy, comprising:
introducing a SATAC that comprises DNA therapeutic product into
20 a target cell; and

introducing the resulting target cells into a host animal.

74. The method of claim 73, wherein the target cells are lymphocytes, stem cells, nerve cells, insect cells, chicken cells or muscle cells.

25 75. The method of claim 73, wherein the minichromosome is the minichromosome present in the cell line EC3/7C5.

76. The method of claim 73, wherein the chromosome is the λ neo-chromosome in the cell line KE1 2/4.

77. The artificial chromosome of claim 76 that is between about
30 20 Mb and about 200 Mb.

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78. The artificial chromosome of claim 76 that is between about 100 Mb and about 200 Mb.

79. The artificial chromosome of claim 76 that is between about 20 Mb and about 200 Mb.

5 80. The artificial chromosome of claim 76 that is between about 1 Mb and about 15 Mb.

81. The method of claim 51, wherein the animal is a mammal or a oviparous animal and the SATAC includes proteins and regulatory elements for expression of genes in the milk of the animal or in the egg of
10 the animal.

82. The method of claim 81, wherein the animal is selected from among cows, goats, oxen, pigs and sheep.

83. The method of claim 81, wherein the animal is selected from among fowl.

15 84. The method of claim 51, wherein the SATAC includes DNA encoding genes for expression of human cell surface proteins, whereby the organs of the animal express the human proteins and will not be rejected upon transplantation into a human.

85. Isolated DNA, comprising the DNA having the sequence set
20 forth in SEQ ID NO. 13, 14 or 15.

86. An isolated DNA, comprising the DNA having the sequence set forth in SEQ ID NO. 13, 14 or 15.

87. An isolated DNA fragment, comprising a sequence of nucleotides set forth in any of SEQ ID Nos. 18-24.

25 88. A SATAC of claim 14, comprising a sequence of nucleotides set forth in any of SEQ ID Nos. 18-24.

89. A SATAC of claim 13, comprising a sequence of nucleotides set forth in any of SEQ ID Nos. 18-24.

90. A SATAC of claim 13, comprising a sequence of nucleotides
30 set forth in any of SEQ ID Nos. 18-24.

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91. A cellular production system, comprising a cell containing an artificial chromosome [AC], wherein the AC comprises multiple copies of a heterologous gene or a plurality of heterologous genes.

92. The cellular production system of claim 91, wherein the AC
5 is a SATAC.

93. The system of claim 91 or claim 92, wherein the heterologous genes encode proteins that comprise a metabolic pathway.

94. A method of expression of a product that is produced upon expression of a metabolic pathway, comprising culturing the system of
10 claim 93 under conditions whereby the proteins comprising the pathway are expressed to produce the product.

95. The method of claim 94, wherein the product is a vitamin, a hormone, a nucleotide, an amino acid, a protein or a peptide.

96. The method of claim 51, wherein the animal is oviparous.
15 97. The method of claim 51, wherein animal is a chicken.

98. The method of claim 51, wherein the animal is an insect.

99. A method for producing a transgenic plant, comprising introducing a satellite artificial chromosome [SATAC] of any of claims 13-18 or 88-90 into a plant cell; and culturing the cell under conditions
20 whereby a plant is generated.

100. The method of claim 99, wherein the SATAC is introduced by protoplast fusion, microinjection, microcell fusion, lipid-mediated gene transfer, electroporation, microprojectile bombardment or direct DNA transfer.

25 101. A method for producing a gene product(s), comprising introducing a satellite artificial chromosome [SATAC] of any of claims 13-18 or 88-90 into a cell; and culturing the cell under conditions whereby the gene product(s) is (are) expressed.

102. The method of claim 102, wherein the gene product is
30 produced by expression of a series of genes that encode proteins that

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comprise a metabolic pathway; and the SATAC comprises each of these genes.

103. An in vitro synthesized artificial mammalian chromosome (ISMAC), comprising a centromere, a telomere, a megareplicator, and a
5 selectable marker, wherein the centromere is derived from a SATAC of any of claims 12-18 and 88-90.

104. An in vitro synthesized artificial mammalian chromosome (ISMAC), comprising a centromere, a telomere, a megareplicator, and a selectable marker, wherein the centromere is derived from a SATAC of
10 any of claims 12-18, and 88-90.

105. The ISMAC of claim 103 or claim 104, further comprising heterochromatin.

106. The ISMAC of any of claims 103-105, wherein the megareplicator comprises rDNA.

15 107. The ISMAC of any of claims 103-106, wherein the centromere is a human centromere.

108. The ISMAC of any of claims 103-107, wherein the centromere is derived from a megachromosome.

109. The ISMAC of any of claims 103, 105, 106 or 108, wherein
20 the centromere is derived from a cell line having all of the identifying characteristics of the cell line deposited under at the European Collection of Animal Cell Culture (ECACC) under Accession No. 96040929.

110. The method of claim 54, wherein the product is a hormone, antibody, cytokine, growth factor, regulatory protein, secretable proteins.

25 111. The method of claim 54, wherein the product is the cystic fibrosis transmembrane regulatory protein [CFTR], an anti-HIV ribozyme, or a tumor suppressor gene.

112. The method of claim 66, wherein the animal is a human.

113. A method of producing an in vitro synthesized artificial
30 mammalian chromosome (ISMAC) comprising, combining a centromer , a

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telomere, a megareplicator, and a selectable marker to produce a replicable ISMAC, herein the centromere is derived from a SATAC of any of claims 12-18, and 88-90.

114. The method of claim 113, further comprising including rDNA
5 in the ISMAC.

115. The method of claim 113, wherein the telomere comprises a plurality of repeats of SEQ ID No. 29.

116. The method of claim 115, wherein the telomere is about 1 kB up about 1 Mb, preferably about 1 kB up to about 500 kB.

10 117. The ISMAC of claim 103-108, wherein the telomere comprises a plurality of repeats of SEQ ID No. 29.

118. The ISMAC of claim 117, wherein the telomere is about 1 kB up to about 1 Mb, preferably about 1 kB up to about 500 kB.

119. A method for producing an artificial chromosome,
15 comprising:

introducing a DNA fragment into a cell, wherein the DNA fragment comprises a selectable marker;

growing the cell under selective conditions to produce cells that have incorporated the DNA fragment into their genomic DNA,

20 wherein the DNA fragment is introduced into or adjacent to an amplifiable region of a chromosome in the cell, whereby a minichromosome comprising the DNA derived from the amplifiable region is produced, wherein the minichromosome is an artificial chromosome that contains more euchromatin than heterochromatin.

25 120. The method of claim 119, wherein the amplifiable region is rDNA.

121. The method of claim 119 or 120, further comprising isolating the minichromosome.

122. A minichromosome produced by the methods of any of
30 claims 119-121.

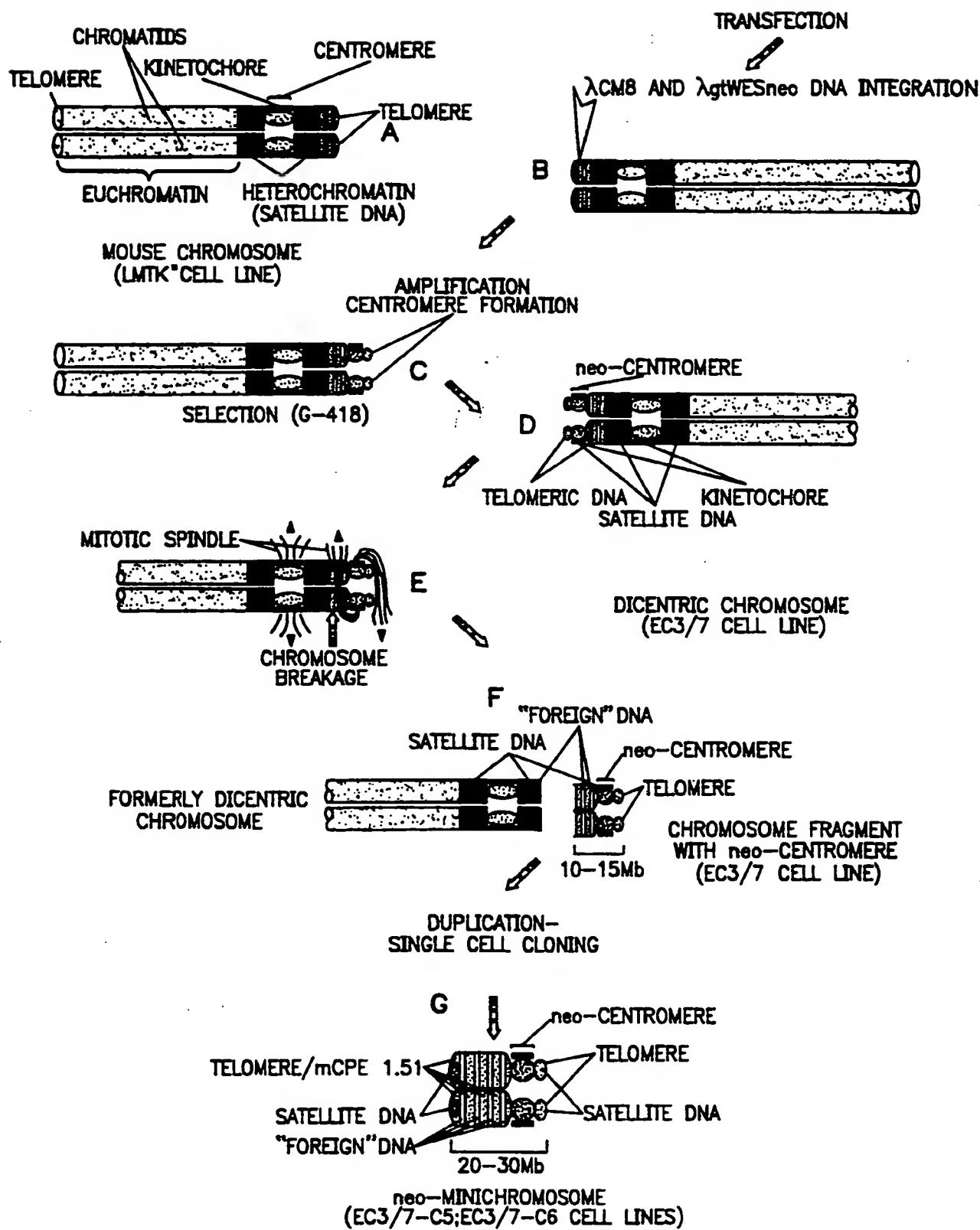
-242-

123. The method of claim 1, further comprising introducing DNA encoding a gene product, wherein:

the DNA encoding the gene is on the fragment that comprises the selectable marker or is on a second DNA fragment; and the resulting

5 SATAc comprises the heterologous DNA that encodes a gene product.

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**FIG. 1**

2 / 5

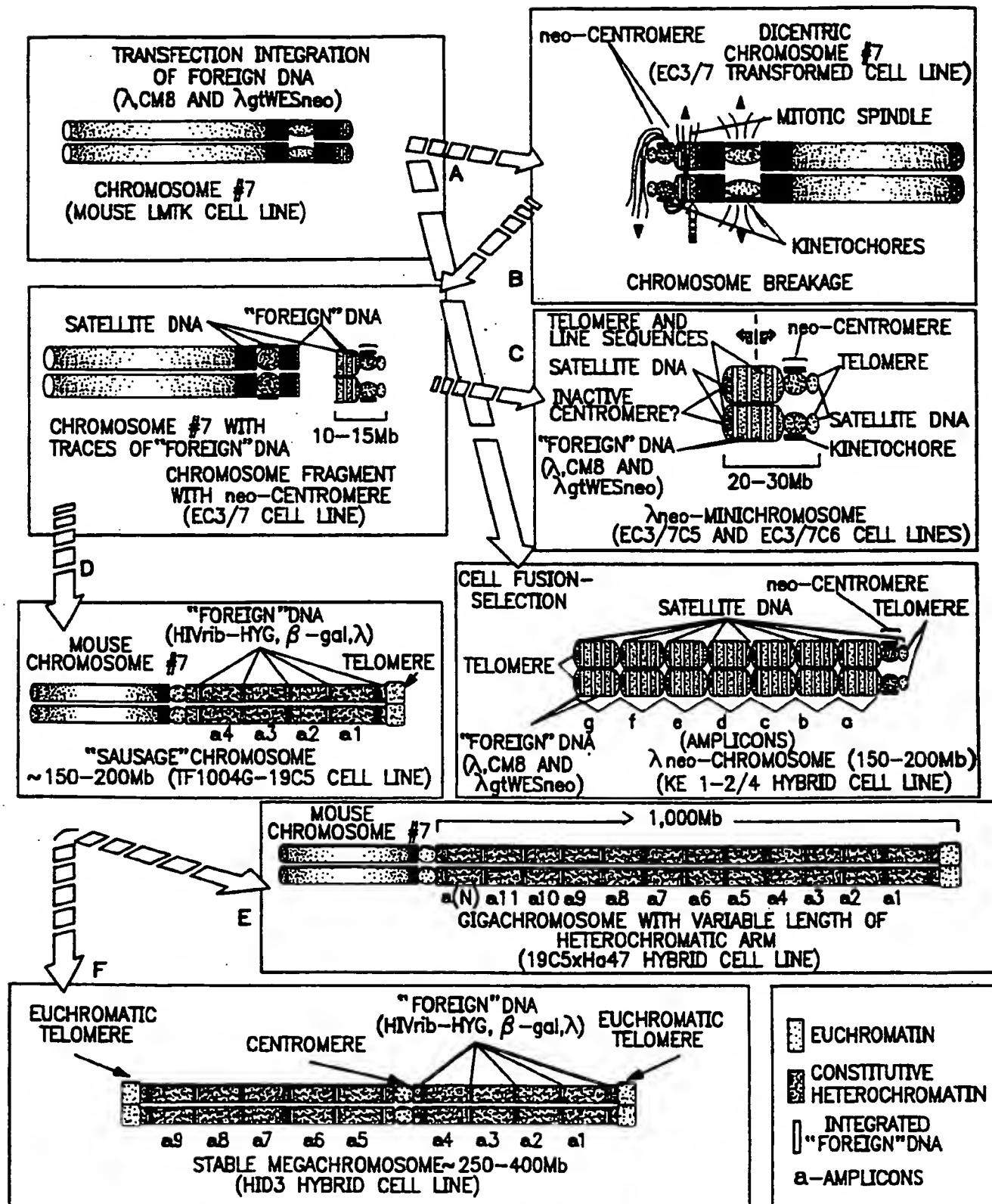
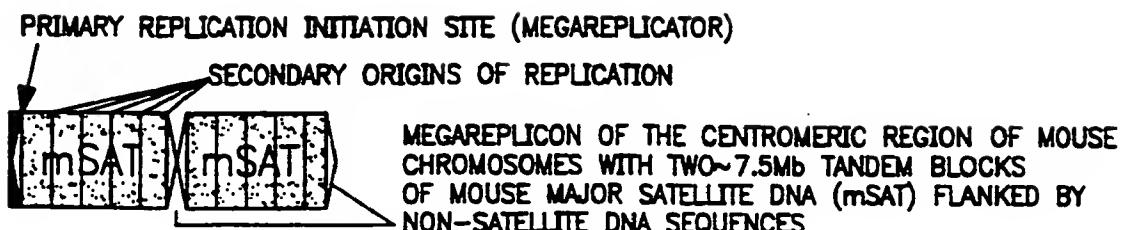


FIG. 2



REPLICATION ERROR GENERATES INVERTED MEGAREPLICONS



AMPLIFICATION PRODUCES A TANDEM ARRAY OF IDENTICAL CHROMOSOME SEGMENTS (AMPLICONS) THAT CONTAIN TWO INVERTED MEGAREPLICONS BORDERED BY THE HETEROLOGOUS ("FOREIGN") DNA

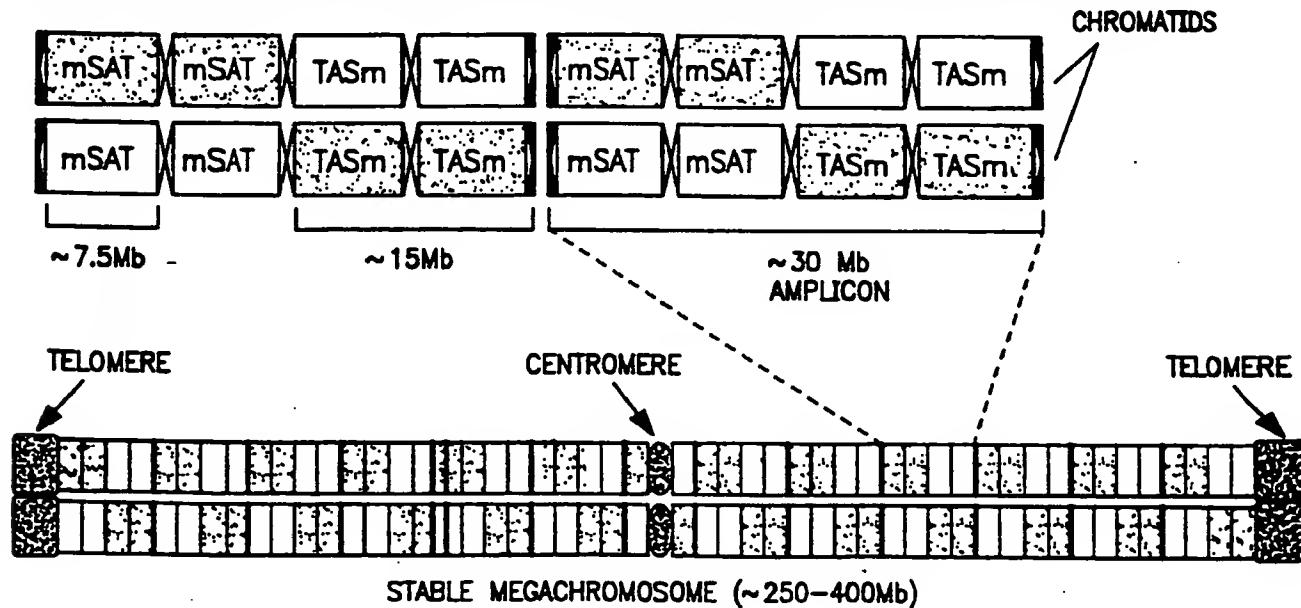
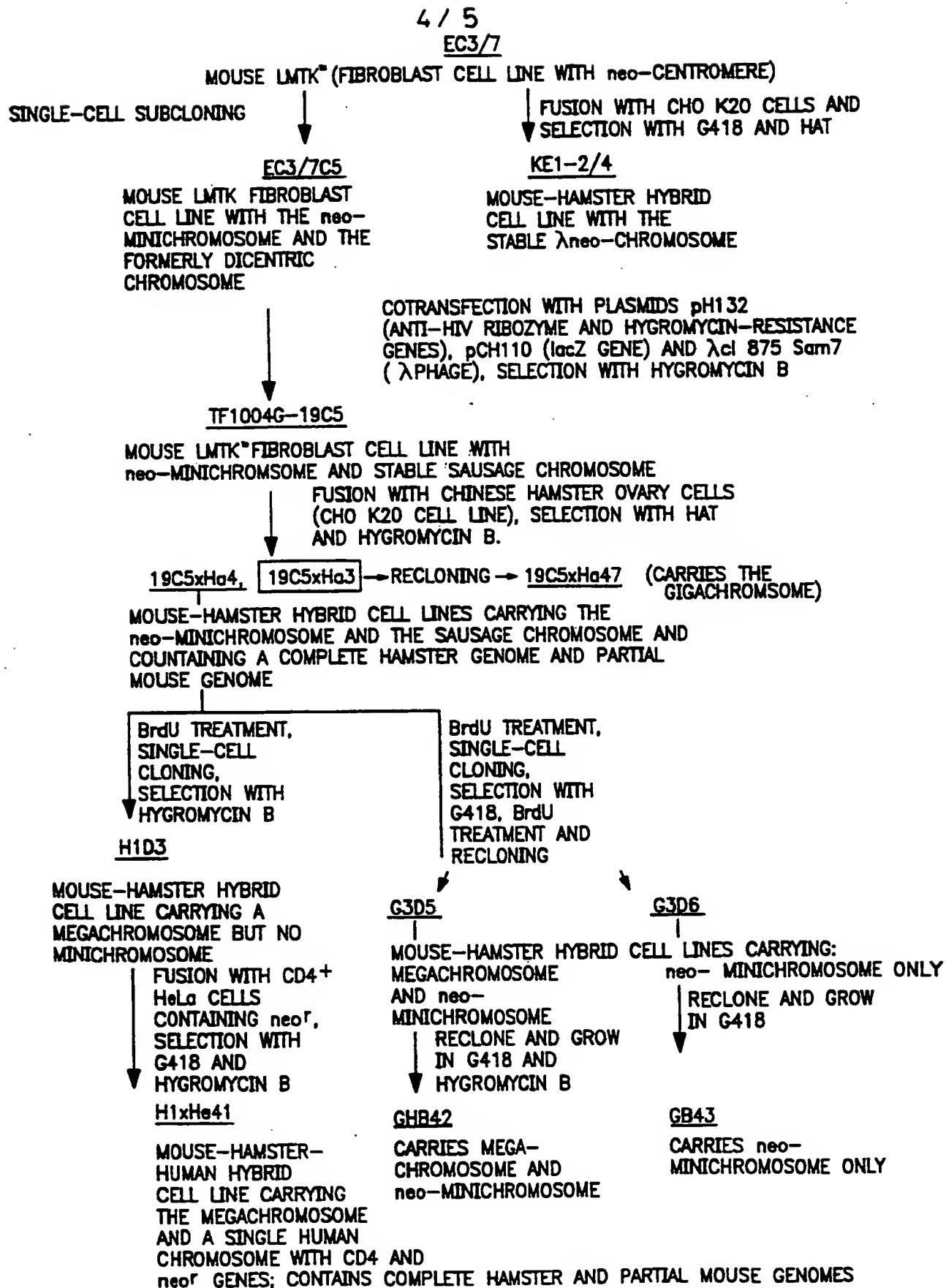
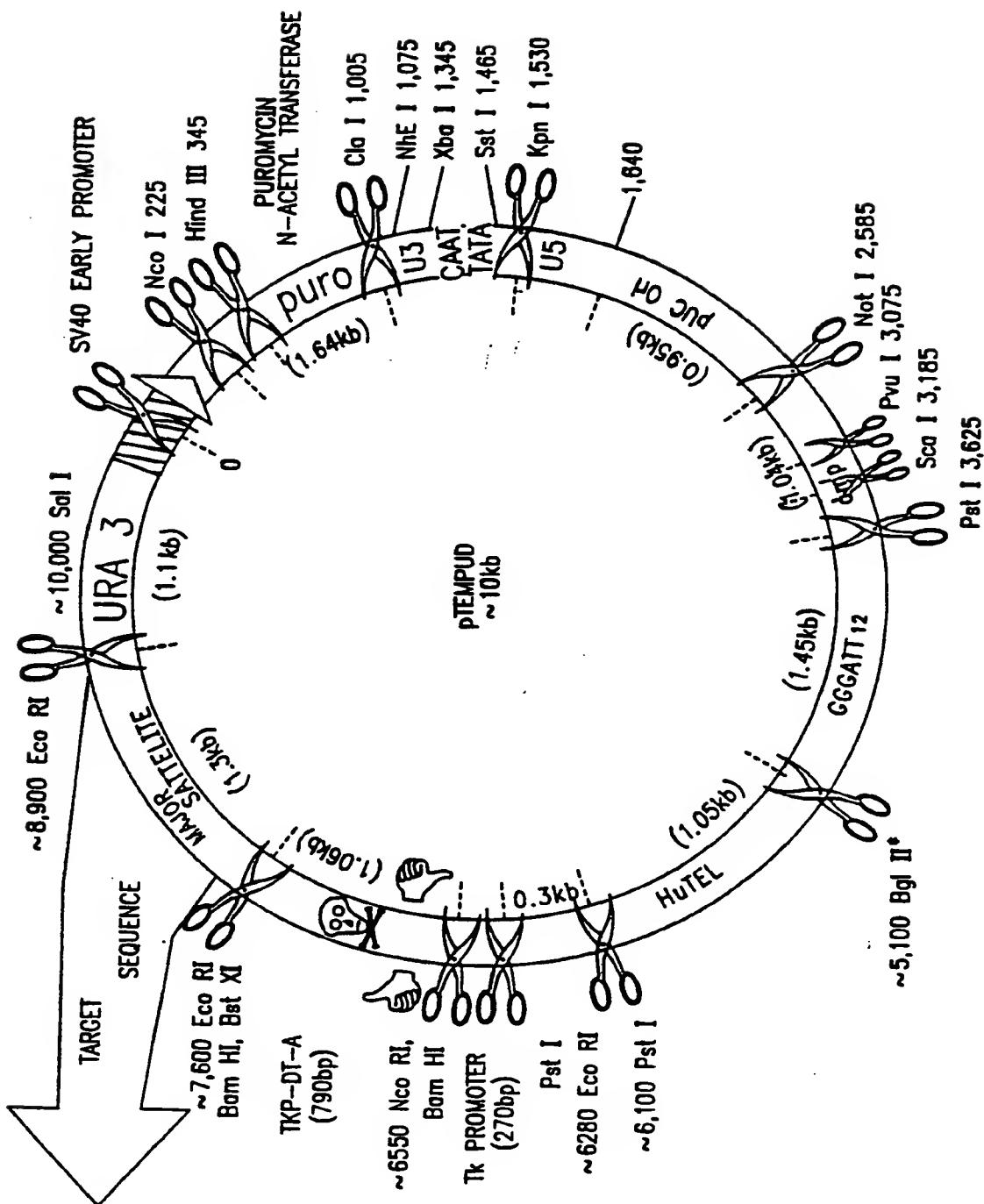


FIG. 3

**FIG. 4**



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682,080	15 July 1996 (15.07.96)	US	
695,191	7 August 1996 (07.08.96)	US	
(71) Applicants (for all designated States except US): THE BIOLOGICAL RESEARCH CENTER OF THE HUNGARIAN ACADEMY OF SCIENCES [HU/HU]; P.O. Box 521, H-6701 Szeged (HU), LOMA LINDA UNIVERSITY [US/US]; Loma Linda, CA 92350 (US). AMERICAN GENE THERAPY, INC. [CA/CA]; 5022 154th Street, Edmonton, Alberta T6H 5P6 (CA).			
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(54) Title: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

(57) Abstract

Methods for preparing cell lines that contain artificial chromosomes, methods for preparation of artificial chromosomes, methods for purification of artificial chromosomes, methods for targeted insertion of heterologous DNA into artificial chromosomes, and methods for delivery of the chromosomes to selected cells and tissues are provided. Also provided are cell lines for use in the methods, and cell lines and chromosomes produced by the methods. In particular, satellite artificial chromosomes that, except for inserted heterologous DNA, are substantially composed of heterochromatin, are provided. Methods for use of the artificial chromosomes, including for gene therapy, production of gene products and production of transgenic plants and animals are also provided.

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

Application No.

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